Novel Cell Lineages Controlled by *Pox neuro* of Larval Poly-Innervated External Sensory Organs in *Drosophila*

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Promotionskomitee

Prof. Dr. Markus Noll

(Vorsitz und Leitung der Dissertation)

Prof. Dr. Konrad Basler

Prof. Dr. Reinhard F. Stocker

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Summary

Drosophila has two basic types of external sensory organs: mono-innervated external sensory (m-es) organs, which are innervated by a single neuron, and poly-innervated external sensory (p-es) organs, which are innervated by multiple, usually two to four, neurons. The *Pox neuro* (*Poxn*) gene, a member of the *Drosophila* Pax gene family, encodes a transcription factor containing a DNA-binding paired domain. *Poxn* plays an important role in the determination of larval p-es organs as well as their adult homologs, the chemosensory bristles. In the absence of *Poxn*, the larval p-es organs and the adult chemosensory bristles are transformed into papilla-like organs and mechanosensory-like bristles, respectively. $Poxn^{\Delta M22}$, a null allele of *Poxn*, was generated in our lab. Homozygous $Poxn^{\Delta M22}$ flies are viable but males are sterile.

How *Poxn* exerts its function during the specification of p-es organs is incompletely understood. To address this question, we analyzed the cell lineages of two larval p-es organs, kölbchen and papilla 6 (p6), in wild-type embryos, as well as the kölbchen lineage in homozygous $Poxn^{\Delta M22}$ embryos, using a temporally and spatially controlled *flp*/FRT recombination system and the Gal4/UAS reporter system.

It is shown that in wild-type embryos the kölbchen lineage gives rise to seven cells, including three support cells, three bipolar neurons, and one multidendritic (md) neuron. The sensory organ precursor (SOP) cell divides to give rise to two secondary precursors, the pIIa and pIIb cell. All support cells are generated from the pIIa cell, whereas all neurons are produced from the pIIb cell. Unexpectedly, the kölbchen lineage is distinct from any cell lineage of sensory organs proposed previously. The most striking difference is that the sheath cell, which in the m-es lineage is generated from the pIIb cell with the bipolar and md neuron, derives from the pIIa cell together with the other support cells.

In homozygous *Poxn*$^{\Delta M22}$ embryos, the kölbchen lineage gives rise to a reduced number of cells that include all support cells but fewer neurons, and most important, the cell division pattern is changed: the sheath cell, like the neurons, is derived from the pIIb cell. We also observed two alternative cell lineages for kölbchen in *Poxn*$^{\Delta M22}$ embryos: in about 65% of the cases five cells are generated, while in 35% of the cases six cells are produced. In the case of five cells, the lineage is completely transformed into the m-es lineage, whereas the lineage produces an additional bipolar neuron when six cells are produced, but the cell division pattern is similar to that of the m-es lineage. We also found that the reduced number of cells does not result from apoptosis.

To test whether the lineage described above is kölbchen-specific, we analyzed the cell lineage of another larval p-es organ, that of papilla p6. The p6 lineage is similar to the kölbchen lineage: all support cells are generated from the pIIa cell while all neurons are derived from the pIIb cell. However, the p6 lineage also exhibits differences in several aspects. First, the SOP of p6 and the precursor cell of a presumptive epidermal gland are derived from one precursor; second, in the p6 lineage, the pIIb cell produces two bipolar neurons, while three bipolar neurons are generated in the kölbchen lineage; third, neither of the two bipolar neurons expresses *Poxn* during late embryogenesis, but one of them expresses BarH1; and last, in the p6 lineage, but not in that of kölbchen, one cell undergoes apoptosis.

In summary, we describe in this study novel cell lineages for two larval p-es organs. Moreover, our loss-of-function studies show that the p-es lineages are affected in the absence of *Poxn*. Our results indicate that *Poxn* plays an important role in the specification of the cell lineages during the development of the p-es organs in *Drosophila* larvae.

Zusammenfassung

Die Taufliege *Drosophila* besitzt zwei Grundtypen von externen sensorischen Organen: Mono-innervierte externe sensorische (m-es) Organe, welche von einem einzelnen Neuron, beziehungsweise poly-innervierte externe sensorische (p-es) Organe, welche von zwei bis vier Neuronen innerviert werden. Das *Pox neuro* (*Poxn*) Gen, ein Mitglied der Pax-Genfamilie von *Drosophila*, kodiert für einen Transkriptionsfaktor mit einer DNA-bindenden paired-Domäne. *Poxn* spielt eine wichtige Rolle bei der Determination der larvalen p-es Organe, aber auch der homologen Organe im Imago, den chemosensorischen Borsten. Beim Fehlen von *Poxn* werden sowohl die larvalen p-es Organe, als auch die chemosensorischen Borsten im adulten Tier in Strukturen transformiert, die in der Larve Papillen, im Imago mechanosensorischen Borsten ähnlich sind. $Poxn^{\Delta M22}$, ein Nullallel von *Poxn,* wurde in unserem Labor hergestellt. Homozygote $Poxn^{\Delta M22}$ Mutanten sind lebensfähig, aber die Männchen sind steril.

Die Funktion von *Poxn* bei der Spezifikation der p-es Organe ist nicht vollständig geklärt. Um dieser Frage nachzugehen, haben wir den Zellstammbaum zweier p-es Organe, des Kölbchens und der Papilla p6 im Wildtyp, den Kölbchen-Zellstammbaum auch im homozygoten *Poxn* Embryo mittels zeitlich und räumlich kontrollierter *flp*/FRT-induzierter Rekombination und dem Gal4/UAS Reportersystem analysiert.

Es wird gezeigt, dass in wildtypischen Embryonen der Zellstammbaum des Kölbchens zu sieben Zellen führt, drei Supportzellen, drei bipolaren Neuronen und einem multidendritischen Neuron. Die sensorische Mutterzelle (SOP) teilt sich und erzeugt zwei sekundäre Vorläuferzellen, pIIa und pIIb. Alle Supportzellen entstehen aus der pIIa-Zelle, während alle Neuronen von der pIIb-Zelle abstammen. Damit ist der Zellstammbaum des Kölbchens überraschenderweise verschieden von allen anderen, bisher beschriebenen

Teilungsmustern für sensorische Organe. Dabei stellt die Abstammung der Begleitzelle (Thecogen) den wohl bemerkenswertesten Unterschied dar: Sie wird nicht, wie im m-es Zellstammbaum, aus der pIIb-Zelle zusammen mit dem Neuron gebildet, sondern entsteht zusammen mit den anderen Supportzellen aus der pIIa Vorläuferzelle.

Im homozygoten *Poxn*$^{\Delta M22}$ Embryo wird im Kölbchen-Zellstammbaum eine reduzierte Anzahl Zellen erzeugt. Aus der asymmetrischen Zellteilungsabfolge entstehen zwar alle Supportzellen, aber nur eine reduzierte Anzahl Neuronen. Dabei ist bemerkenswert, dass die Begleitzelle zusammen mit den Neuronen aus der pIIb-Zelle erzeugt wird. Wir haben zwei leicht unterschiedliche Zellstammbäume für Kölbchen in *Poxn*$^{\Delta M22}$ Embryos beobachtet: In etwa 65% der Fälle werden fünf Zellen gebildet, in 35% der Kölbchen sind es aber sechs Zellen. In beiden Fällen entspricht der Zellstammbaum vollständig dem m-es Teilungsmuster, in etwa einem Drittel der Fälle wird aber zusätzlich ein zweites bipolares Neuron erzeugt. Wir haben auch festgestellt, dass die reduzierte Anzahl der Zellen im Stammbaum nicht durch Apoptose verursacht wird.

Um zu testen, ob der oben beschriebene Zellstammbaum Kölbchen-spezifisch ist, haben wir das Teilungsmuster eines anderen p-es Organs, nämlich der Papilla p6 analysiert. Der p6-Zellstammbaum ist dem des Kölbchens sehr ähnlich: Alle Supportzellen entstehen aus der pIIa-Vorläuferzelle, während alle Neuronen von pIIb abstammen. Der p6-Stammbaum weist aber einige Unterschiede auf. Erstens wird die sensorische Mutterzelle, zusammen mit der Vorläuferzelle einer mutmasslichen, epidermalen Drüse, aus einer gemeinsamen Vorläuferzelle gebildet; zweitens werden in der p6-Linie von der pIIb-Tochterzelle nur zwei und nicht wie in der Kölbchenlinie drei bipolare Neuronen gebildet; drittens exprimiert keines der zwei bipolaren Neuronen *Poxn* in der späten Phase der Embryogenese, aber eines BarH1; und zuletzt wird eine Zelle in der p6-Linie, aber nicht in derjenigen des Kölbchens durch Apoptose eliminiert.

Zusammenfassend beschreiben wir in dieser Arbeit neue Zellstammbäume für zwei larvale p-es Organe. Zusätzlich zeigen unsere Untersuchungen an der amorphen Mutante, dass die p-es Zellstammbäume durch den Verlust der *Poxn* Funktion beeinträchtigt sind. Unsere Resultate weisen darauf hin, dass *Poxn* eine wichtige Rolle in der Spezifikation der Zellstammbäume während der Entwicklung der p-es Organe in der *Drosophila* Larve spielt.

Chapter 1

General Introduction

I. The peripheral nervous system of *Drosophila*

A fundamental aspect in the development of multicellular organisms is the determination of different cell fates. However, the mechanism by which undifferentiated cells adopt specific cell fates during development is incompletely understood. Cell fate specification is particularly intriguing during neurogenesis, which gives rise to an astonishing diversity of cell types that form extremely complex networks and connections with high precision.

A well-established system to investigate cell fate determination is the peripheral nervous system (PNS) of *Drosophila* (Jan and Jan, 1993; Jan and Jan, 1994; Bellaïche and Schweisguth, 2001; Betschinger and Knoblich, 2004; Lai and Orgogozo, 2004). *Drosophila* has both a larval and an adult PNS. The larval PNS is formed during embryogenesis, and the majority of the larval PNS degenerates during metamorphosis. The adult PNS forms de novo during pupal development (Jan and Jan, 1993).

The larval PNS includes external sensory organs as well as internal sensory organs, such as chordotonal organs and multidendritic (md) neurons (Jan and Jan, 1993). External sensory organs consist of two basic types: mono-innervated external sensory (m-es) organs, which are innervated by a single bipolar neuron, and poly-innervated external sensory (p-es) organs, which consist of multiple, usually two to four, bipolar neurons (Ghysen et al., 1986; Hartenstein, 1988; Campos-Ortega and Hartenstein, 1997).

The larval external sensory organs are organized in a stereotyped pattern (Fig. 1A) (Hertweck, 1931; Dambly-Chaudière and Ghysen, 1986). While most external sensory organs are m-es organs that are either a dome-like campaniform sensillum (papilla) (Fig. 1E)

or a bristle-like trichoid sensillum (hair) (Fig. 1C, D), two p-es organs are present in each thoracic and abdominal hemisegment. The p-es organs of the thoracic segments are the peg-like basiconical sensilla (kölbchen) (Fig. 1B), which are located in a ventral and dorsal (in the prothoracic segment T1) or in a ventral and lateral position (in the meso- and metathoracic segments T2 and T3). The p-es organs of the first seven abdominal segments (A1-A7) are the ventrolateral papilla p6 and the dorsal hair h3 (Fig. 1D, arrow) (Dambly-Chaudière and Ghysen, 1986).

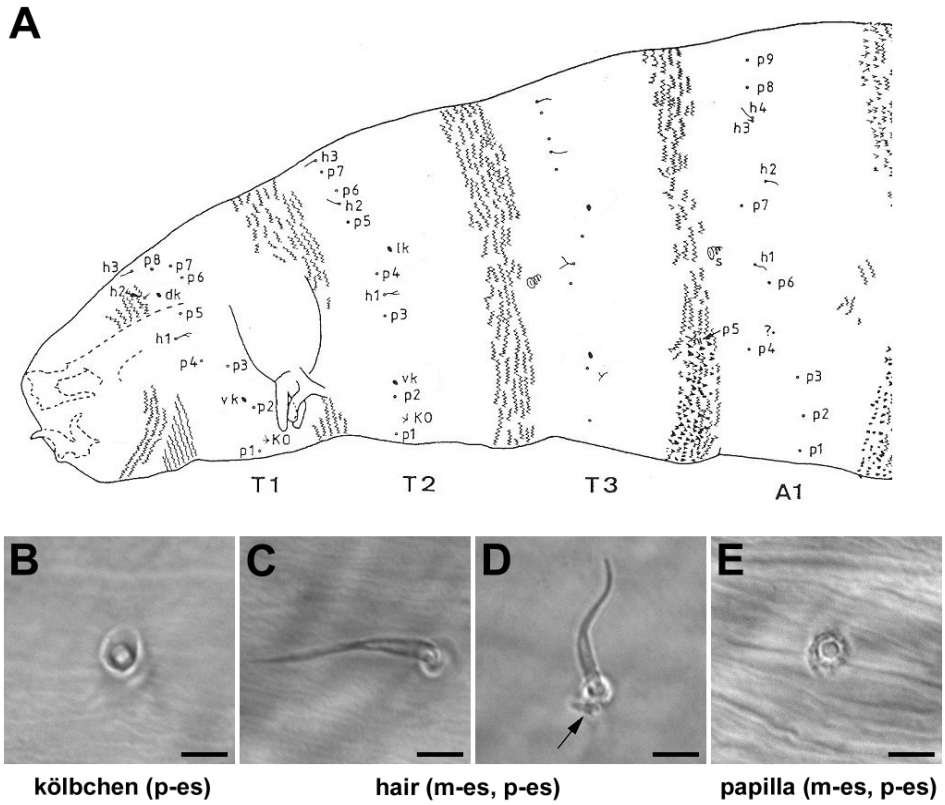

Fig.1. External sensory organs of *Drosophila* larvae. **(A)** Drawing of the anterior end of a third instar larva with labeled external sensory organs in the pro- and mesothoracic and first abdominal hemisegments (from Dambly-Chaudière and Ghysen, 1986). **(B-E)** Different types of external sensory organs of a third instar *y w* larva, including a kölbchen (B), a hair (C), the very short p-es hair h3 (arrow) and the neighboring m-es hair h4 (D), and an m-es or p-es papilla (E). dk: dorsal kölbchen; lk: lateral kölbchen; vk: ventral kölbchen; h: hair; p: papilla; KO: Keilin's organ; s: residual spiracles; and the question mark in A1 segment labels a very small pit that might be an epidermal gland. Scale bars: 5µm.

The exact functions of the larval external sensory organs are not known. The m-es organs are believed to be mechanosensory organs, as mechanosensory organs typically possess a single sensory neuron. The p-es organs are proposed to be chemosensory organs, or organs with both chemosensory and mechanosensory modalities. Based on morphological similarities to electrophysiologically defined sensory organs in other insects, kölbchen have been suggested to be combined thermosensory and hygrosensory organs (Hartenstein, 1988). Moreover, one of the candidate gustatory receptors in *Drosophila*, Gr2B1, has been shown to be expressed in one of the neurons innervating the ventral kölbchen, which suggests that kölbchen may play a role in taste perception (Scott et al., 2001). Recently, two atypical soluble guanylyl cyclases were characterized in *Drosophila*. These enzymes are activated by nitric oxide and are expressed in a subset of peripheral sensory neurons, including one neuron that innervates kölbchen (Langlais et al., 2004). All these findings indicate that kölbchen might be involved in chemosensory perception.

The adult PNS also includes mono-innervated mechanosensory sensilla as well as poly-innervated chemosensory sensilla. The chemosensory system of adult *Drosophila* has been described in detail by Stocker (Stocker, 1994) and reviewed recently by Vosshall and Stocker (Vosshall and Stocker, 2007).

II. The development of larval external sensory organs

The larval external and internal sensory organs are derived from sensory organ precursor (SOP) cells. During early neurogenesis, groups of cells in the neuroectoderm acquire the potential to become neuronal precursor cells by the expression of proneural genes, such as the genes of the *achaete-scute* complex (Fig. 2A). From each group a single cell is selected to become a neuronal precursor through a process called “lateral inhibition” (Fig. 2A), which is mediated by the neurogenic genes like *Notch* and *Delta*, which silence the proneural genes

through the Notch (N) signaling pathway in all cells of the proneural group except the SOP. The identity of an SOP cell is further determined by the action of neuronal precursor genes and neuronal precursor-type selector genes that are required to initiate the precise developmental program of a particular sensory organ (Fig. 2A) (Jan and Jan, 1993).

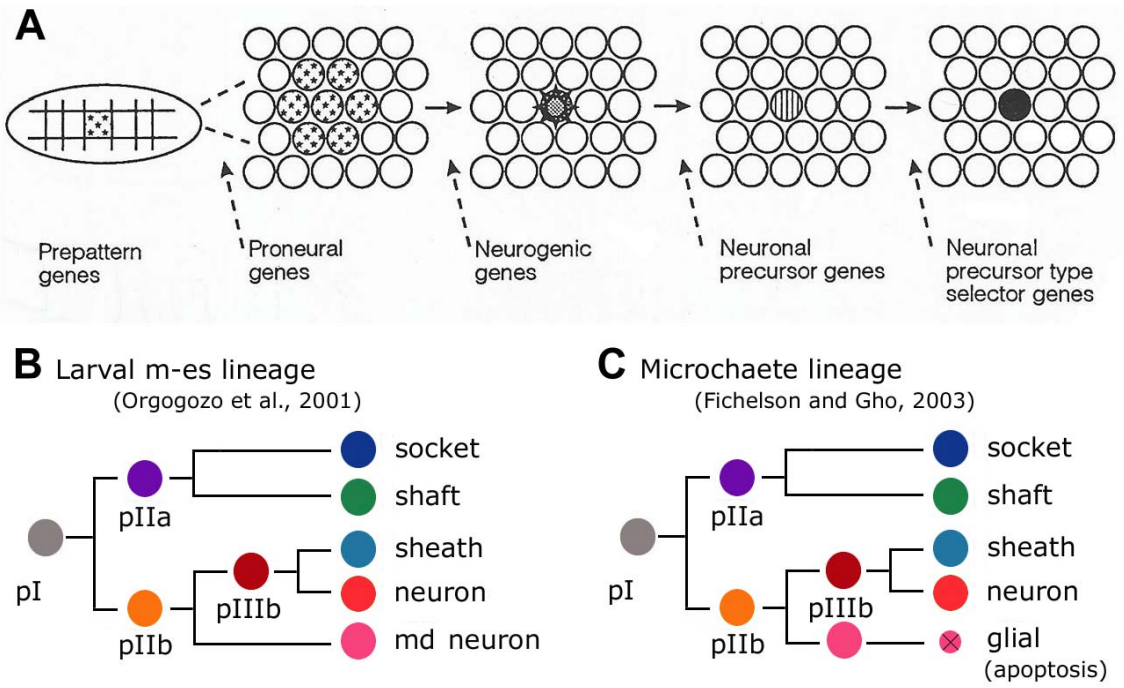

Fig. 2. Development of external sensory organs in *Drosophila*. **(A)** Progressive determination of the SOP cell during embryogenesis (from Jan and Jan, 1993). **(B)** Cell lineage of larval m-es organs. The SOP or pI cell divides to give rise to the two secondary precursors, pIIa and pIIb. The pIIa is the precursor of the socket and shaft cell, while the pIIb cell produces an md neuron and the pIIIb cell that will further divide to generate the sheath cell and one bipolar neuron. **(C)** Cell lineage of a microchaete in adult *Drosophila*. The cell division pattern of the microchaete lineage is very similar to that of the larval m-es lineage, except that the glial cell generated from the pIIb cell undergoes apoptosis shortly after birth.

Once an SOP cell is determined, it will follow a cell lineage consisting of a series of asymmetric cell divisions to give rise to a number of daughter cells, including the external sensory organ cells (three support cells and one or multiple bipolar neurons) and one md neuron (Bodmer et al., 1989; Brewster and Bodmer, 1995; Orgogozo et al., 2001; Lai and

Orgogozo, 2004). Asymmetric cell division is controlled by cell-autonomous mechanisms as well as cell-cell communication between sibling cells. Cell-autonomous regulation is achieved through the asymmetric segregation of cell fate determinants, e.g., Numb and Neuralized, into only one of the two daughter cells after mitosis (Rhyu et al., 1994; Le Borgne and Schweisguth, 2003), and cell-cell communication is regulated by the N signaling pathway (Guo et al., 1996). Through the combination of cell-autonomous mechanisms and cell-cell interactions each individual cell obtains its specific fate and differentiates into the correct cell type.

Of these cell lineages the larval m-es lineage is best characterized (Orgogozo et al., 2001). The SOP cell divides first to give rise to two daughter cells, the secondary precursor pIIa and pIIb (Fig. 2B). The pIIa cell is the precursor of the socket cell and shaft cell, and the pIIb cell divides to generate an md neuron and the pIIIb cell, which divides once more to produce the sheath cell and a bipolar neuron (Fig. 2B). For p-es organs, it has been proposed that the pIIIb cell goes through additional cell divisions to produce multiple neurons (Jan and Jan, 1993; Brewster and Bodmer, 1995).

Several cell lineages for both mechanosensory and chemosensory sensilla in the adult have also been described (Hartenstein and Posakony, 1989; Ray et al., 1993; Gho et al., 1999; Reddy and Rodrigues, 1999; Fichelson and Gho, 2003). All these lineages are similar to the larval m-es lineage. Among them, an interesting one is the microchaete linage (Fichelson and Gho, 2003). In this lineage, the pIIb cell gives rise to the pIIIb cell and a glial cell, and the latter undergoes programmed cell death soon after birth (Fig. 2C).

The internal sensory organs are also generated by defined cell lineages that have been described in detail previously (Bodmer et al., 1989; Brewster and Bodmer, 1995; Orgogozo et al., 2002; Lai and Orgogozo, 2004).

III. Pax genes and *Pox neuro*

The *Pax* (*pa*ired bo*x*) genes encode a family of highly conserved transcription factors that contain a characteristic 128-amino acid DNA-binding paired domain (Noll, 1993). The first Pax gene, *paired* (*prd*), was isolated from *Drosophila* (Bopp et al., 1986). In succession, other members of this gene family in *Drosophila* were identified (Bopp et al., 1986; Bopp et al., 1989; Quiring et al., 1994; Fu and Noll, 1997; Czerny et al., 1999). Homologs of *Drosophila* Pax genes have been characterized in numerous species from sponges, cnidaria, nematodes, and insects to vertebrates (Noll, 1993; Stuart and Gruss, 1995; Sun et al., 1997; Hoshiyama et al., 1998; Hobert and Ruvkun, 1999). In addition to the paired domain, many Pax proteins also contain a *prd*-type homeodomain and/or an octapeptide motif (Noll, 1993). At present, nine mammalian Pax genes (*Pax1* to *Pax9*) have been identified. Based on the similarities in the coding sequences and gene structure, they have been divided into four subgroups: *Pax 1*/*9*, *Pax 2*/*5*/*8*, *Pax 3*/*7*, and *Pax 4*/*6* (Noll, 1993; Stuart and Gruss, 1995; Chi and Epstein, 2002).

Pax genes exhibit highly dynamic expression patterns in a large variety of tissues during animal development and play crucial roles in morphogenesis and organogenesis. Mutations in Pax genes or mis-regulation of the expression of Pax genes not only cause severe developmental defects, but are also associated with a number of human diseases like the Waardenburg's syndrome type I, and furthermore can lead to tumor formation (Noll, 1993; Stuart and Gruss, 1995; Chi and Epstein, 2002).

The *Pox neuro* (*Poxn*) gene, a member of the *Drosophila* Pax gene family, encodes a transcription factor containing a DNA-binding paired domain but no homeodomain (Bopp et al., 1989; Noll, 1993). *Poxn* plays essential roles in many aspects during the development of the PNS in both larvae and adults, where it is expressed in p-es organs as well as a subgroup of m-es organs in a temporally and spatially controlled fashion (Dambly-Chaudière et al.,

1992; Awasaki and Kimura, 1997; Boll and Noll, 2002). In the absence of *Poxn*, the larval p-es organs as well as the adult chemosensory sensilla are transformed to papilla-like organs and mechanosensory-like sensilla, respectively; and the larval m-es hairs have degenerated shafts in the second and third instar. *Poxn* is also expressed in the developing larval and adult brain and in the central nervous system (CNS), plays additional roles in the development of adult appendages, and controls male courtship behavior and fertility (Awasaki and Kimura, 2001; Boll and Noll, 2002). *Poxn* null mutants were generated in our lab. They are viable, but males are sterile (Boll and Noll, 2002).

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Chapter 2

Novel Cell Lineages Controlled by *Pox neuro* of Larval Poly-Innervated External Sensory Organs in *Drosophila*

Materials and Methods

Drosophila strains

The following fly strains were used in this study:

y w

y w; Poxn$^{\Delta M22}$/CyO y^{+}

y w hs-flp; UAS>CD2, y^{+}>CD8:GFP/CyO; TM2/TM6B

w; Poxn-Gal4^{ups1f}/TM3

y w; E7-2-36

y w; ; D-Pax2-Gal4^{Y283}

y w; ; achaete-Gal4^{13}

y w; ; daughterless-Gal4

w; UAS-Poxn

w; ; UAS-Poxn

w; UAS-p35

y w; UAS-CD8:GFP/CyO

w; Poxn$^{\Delta M22}$/CyO; Poxn-CD8:GFP$^{3-3}$

y w hs-flp; UAS>CD2, y^{+}>CD8:GFP/CyO; Poxn-Gal4^{ups1f}/TM6B

y w hs-flp; UAS>CD2, y^{+}>CD8:GFP/CyO; D-Pax2-Gal4^{Y283}

y w hs-flp; Poxn$^{\Delta M22}$ UAS>CD2, y^{+}>CD8:GFP/CyO; Poxn-Gal4^{ups1f}/TM6B

y w hs-flp; E7-2-36 UAS>CD2, y^{+}>CD8:GFP/CyO; Poxn-Gal4^{ups1f}/TM6B

y w hs-flp; Poxn$^{\Delta M22}$ E7-2-36/CyO; Poxn-Gal4^{ups1f}/TM6B

y w hs-flp; Poxn$^{\Delta M22}$ UAS-p35/CyO; Poxn-Gal4^{ups1f}/TM6B

y w; UAS-CD8:GFP; Poxn-Gal4^{ups1f}/TM3

y w hs-flp; UAS>CD2, y^{+}>CD8:GFP/CyO; achaete-Gal4^{13}

y w hs-flp; UAS>CD2, y^+>CD8:GFP/CyO; daughterless-Gal4

Generation of GFP-expressing clones in the embryonic PNS

Flies were allowed to lay eggs on apple juice plates with a small amount of yeast for two hours at 18˚C. The plates were kept at 18˚C for 4, 6, 8, 10, and 12 hours before the embryos were collected in a fine custom-built nylon sieve. Thus, the age of the collected embryos corresponded to 2-3, 3-4, 4-5, 5-6, and 6-7 hours of development at 25°C. Embryos were rinsed several times with cold tap water before they were subjected to heat shock. Heat shock was performed by placing the nylon sieves containing the embryos directly into a pre-heated 32˚C waterbath and keeping the embryos submerged for 30 minutes. Embryos carrying the *D-Pax2-Gal4* transgene were heat-shocked at 30˚C for 25 minutes. After heat shock, embryos were rinsed briefly with cold tap water and transferred, still on the sieves, into a humidified chamber. Embryos were allowed to develop at 18˚C until they reached 14-15 hours of development at 25°C (stage 16) and then were fixed and immunostained.

Antibodies and immunohistochemistry

The following primary antibodies were used: rabbit anti-Poxn (1:50; Bopp et al., 1989); rabbit anti-D-Pax2 (1:100; Fu and Noll, 1997); chicken anti-GFP (1:500; Abcam); mouse anti-CD2 (1:100; Serotec); rabbit anti-Su(H) (1:100; Santa Cruz Biotechnology); mouse anti-Prospero (1:10; batch MR1A, DSHB); mouse anti-22C10 (1:100; DSHB); rabbit anti-β-galactosidase (1:1000; Cappel); rabbit anti-BarH1 (1:100; Higashijima et al., 1992); rabbit anti-cleaved Caspase 3 (Asp175) (1:100; Cell Signaling Technology); rat anti-Elav (1:200; DSHB). The secondary antibody used in Figure 1 was biotinylated goat anti-rabbit IgG (Vector Labs); other secondary antibodies used in this study were: Alexa 488-, Alexa 594-,

and Alexa 647-conjugated anti-chicken, -rabbit, -mouse, or -rat IgG (1:500; all from Molecular Probes).

Embryos were fixed and stained as described previously (Gutjahr et al., 1993; Boll and Noll, 2002).

Preparation of larvae for the observation of external sensory organs

Third instar larvae were collected and washed several times with tap water and transferred to Eppendorf tubes containing a solution of 7% gelatin and 50% glycerol. The tubes were kept at 40˚C for 15-20 minutes. Larvae were then squashed and mounted on glass slides for observation.

Microscopy and image processing

Poxn expression patterns in wild-type embryos were visualized by immunohistochemistry and recorded with a Zeiss Axiophot microscope equipped with a Hamamatsu CCD camera. Sensory organs of third instar larvae were observed with a Zeiss Axiophot microscope equipped with DIC optics. Images of immunofluorescent specimens were acquired with a Leica TCS SP confocal microscope. The resulting Z-stack images were processed with LCS Lite software (Leica) and Photoshop (Adobe Systems). Immunofluorescent images presented in this study are maximal projections of confocal Z-stack images.

Results

I. The embryonic expression pattern of *Poxn*

Poxn exhibits a highly dynamic expression pattern, both temporally and spatially, during embryonic development (Fig. 1). The expression pattern of *Poxn* has been described previously (Dambly-Chaudière et al., 1992). However, we noticed that the reported expression pattern of *Poxn* in the developing PNS was incomplete. In the PNS, *Poxn* expression is first observed at late stage 10, in a single cell in each thoracic and abdominal hemisegment (Fig. 1A). At stage 11 a second cell expressing *Poxn* appears ventrally in each hemisegment (Fig. 1B). It has been shown that these cells are the SOP cells of the larval p-es organs (Dambly-Chaudière et al., 1992). Subsequently, SOP cells start to divide (Fig. 1B), giving rise to two clusters of *Poxn*-expressing cells in each hemisegment (Fig. 1C). During stage 12, the dorsal clusters of *Poxn*-expressing cells in T2 and T3 are located at a more ventral position than that of T1 (Fig. 1C, arrows). At the onset of stage 13, expression of *Poxn* begins to fade in most cells (Fig. 1D). However, in the thoracic segments, one cell in each cluster continues to express *Poxn* very strongly during later stages (Fig. 1E,F; arrowheads in F) until the end of embryogenesis. These single *Poxn*-expressing cells are bipolar neurons innervating the kölbchen.

Poxn is also expressed in several cells of the gnathal segments, in the terminal abdominal segments, in the developing larval brain and the ventral nerve cord, in a few cells associated with the olfactory and gustatory sensory organs of the larval head, in a single cell associated with the larval mono-innervated hairs during late embryogenesis (after stage 14), and in many epidermal cells (Fig. 1A-F).

II. Impaired external sensory organs in the absence of *Poxn*

The embryonic expression pattern suggests a role of *Poxn* during the development of larval external sensory organs, especially the p-es organs. $Poxn^{\Delta M22}$ is a null allele of *Poxn* that was generated in our lab (Boll and Noll, 2002). In homozygous $Poxn^{\Delta M22}$ larvae, all p-es organs as well as a subset of m-es organs in the thoracic and abdominal segments are affected. In the thoracic segments, kölbchen are transformed into papilla-like sensory organs (Fig. 2B). The position of the lateral kölbchen in T2 and T3 is not affected in these animals (compare Fig. 2A with Fig. 2B). The same is true for the dorsal kölbchen of T1 (data not shown). However, the ventral kölbchen are formed more dorsally adjacent to papilla p3 (compare Fig. 2A with Fig. 2B), and in some cases the two organs are even fused with each other (data not shown). In the abdominal segments, the dorsal p-es hair h3 (Fig. 2C) is transformed into a papilla-like sensory organ and is located more ventrally between papilla p7 and hair h2 (Fig. 2F, F'), and sometimes forms a fused organ with p7 (Fig. 2F''). The ventral p-es papilla p6 (Fig. 2G) is found more dorsally close to h1 (Fig. 2H, H'), and sometimes fused with h1 (Fig. 2H''). In some segments, p6 is even transformed into a hair with a degenerated shaft in the second and third instar (Fig. 2H'''). Hairs without shaft are clearly distinguishable from papillae (compare h1 with p6* in Fig. 2H'). In both thoracic and abdominal segments, the m-es hairs are also affected in the absence of *Poxn*. These hairs have lost their shafts during the transition from first to second instar (Fig. 2B'', D, F', H', H''').

The defects of external sensory organs observed in $Poxn^{\Delta M22}$ larvae are in accord with previous analyses of a strong hypomorphic *Poxn* mutant allele, $Poxn^{70}$ (Awasaki and Kimura, 2001), or a large deficiency, *Df(2R)WMG*, uncovering *Poxn* (Dambly-Chaudière et al., 1992).

III. A *flp*-mediated recombination system to study the cell lineage of larval p-es organs

To analyze the cell lineages of larval p-es organs, we designed an assay which combines a temporally controlled *flp*/FRT recombination system (Golic and Lindquist, 1989) with a spatially controlled Gal4/UAS reporter system (Brand and Perrimon, 1993) to generate and label clones derived from SOP cells with cell specific markers (CD2 or CD8:GFP) (Fig. 3). Embryos carrying three transgenes – the yeast recombinase *flp*, whose expression is driven by the heat-inducible *hsp70* promoter (Struhl and Basler, 1993); *Poxn-Gal4*^{ups1f} [*Poxn* transgene *EvK* (Boll and Noll, 2002), whose coding region has been replaced by that of *Gal4* (W. Boll, unpublished)] which expresses Gal4 in all SOPs and their daughter cells of larval p-es organs; and the reporter transgene *UAS>CD2, y*$^{+}$*>CD8:GFP* (Wong et al., 2002) – are subjected to a moderate heat shock. *flp*-mediated recombination between the two FRT sites during mitosis leads to the excision of the *CD2, y*$^{+}$ cassette and brings *CD8:GFP* under the control of Gal4, which activates its UAS enhancer in this cell and all of its progeny (Fig. 3).

To obtain clones resulting from a single recombination event in each lineage, pilot experiments were performed to determine the optimal conditions by varying the temperature and duration of the heat shock. Embryos collected and aged to a stage before cellular blastoderm [corresponding to 2-3 hours after egg laying (AEL) at 25°C] were subjected to a single heat shock and afterwards allowed to develop to stage 16 before fixation and immunostaining (see details in Materials and Methods). Several hundreds of embryos were analyzed by confocal microscopy, and the numbers of GFP-expressing clones were scored in thoracic and abdominal segments A1-A7. Table 1 shows the average number of GFP clones per embryo under five different heat shock conditions. A heat shock at 32°C for 30 minutes produced on average about one thoracic clone per embryo, labeling only one of the twelve kölbchen lineages, which is a good indication that a single recombination event took place in this lineage. Therefore, these heat shock conditions were applied in the following analysis.

Two features of this system facilitated our analysis: (i) all daughter cells of the SOP express *Poxn* and hence *Gal4* of the *Poxn-Gal4* transgene until stage 13, when asymmetric cell division of the lineage has ceased, and (ii) the stability of the Gal4, GFP and CD2 proteins causes perdurance of these markers, which allowed us to analyze the clones at stage 16, when these cells have adopted their final fates and express cell-type specific proteins. The combinatorial analysis of such cell-type specific markers and the distribution pattern of GFP- and CD2-expressing cells within a large number of individual clones, induced at different times after egg laying, enabled us to distinguish all cells within a lineage, quantify each type of clone, and determine the cell division pattern.

IV. Cell lineage of kölbchen in wild-type embryos

To understand the role of *Poxn* during the specification of larval p-es organs, we first analyzed the cell lineage of kölbchen in wild-type embryos. When the embryos were heat-shocked early during embryogenesis (corresponding to 2-3 hours AEL at 25°C), all daughter cells of a precursor of kölbchen are expected to be labeled by GFP, as the SOP cells have not yet been selected by lateral inhibition and will express CD8:GFP if they are derived from a cell that has undergone flp-induced recombination (Fig. 3). Clones induced in both dorsal/lateral kölbchen (dk/lk) and ventral kölbchen (vk) lineages consist of seven cells, which indicates that the kölbchen lineage gives rise to seven cells (Fig. 4A, B). We focused on clones in the dk/lk lineages in our analysis since these cells are better separated from each other and it is much easier to distinguish every single cell by its position (Fig. 4A, A').

Among the seven cells, one cell expresses Suppressor of Hairless [Su(H)], a transcription factor that is expressed in socket cells (Gho et al., 1996). Moreover, this *Su(H)*-expressing cell is always located most dorsally within a clone (Fig. 4C, C'). *D-Pax2*, another paired-domain containing transcription factor of *Drosophila* (Fu and Noll, 1997), is known to be

expressed in shaft and sheath cells (Kavaler et al., 1999). The more ventrally located cell of the two D-Pax2-expressing cells also expresses Prospero (Pros), a sheath cell marker (Vaessin et al., 1991), which suggests that the dorsal D-Pax2-expressing cell is a shaft cell (Fig. 4D, D'). Four cells express *futsch*, which encodes a membrane-associated protein recognized by the monoclonal antibody 22C10 (Zipursky et al., 1984; Hummel et al., 2000), and the nuclear protein Elav (Robinow and White, 1991), both of which are markers specific for neurons, which indicates that the kölbchen lineage generates four neurons (Fig. 4E, E', F, F'). To further determine the identity of the neurons, we utilized the E7-2-36 enhancer trap line that expresses *lacZ* in all md neurons (Bier at al., 1989). As evident from Fig. 4G, G', *lacZ* was always expressed in the neuron located at the most ventral position.

When the embryos were heat-shocked at a slightly later stage during embryogenesis (corresponding to 3-4 hours AEL at 25°C), the majority of the induced clones contained all seven cells. However, in a number of clones, only a subset of the lineage was labeled by GFP, which indicates that the *flp*-induced recombination happened after the division of the SOP (Table 2). When the heat shock was applied at about 4-5 hours AEL, most GFP-expressing clones labeled only part of the kölbchen lineage (Table 2). Mainly two types of clones were observed. The first type contains three cells, of which one cell is always located most dorsally and the other two express D-Pax2, which suggests that these are the three support cells (Fig. 5A, A'). The second type of clone contains four GFP-positive cells. None of them expresses D-Pax2, and all localize ventrally of the CD2-expressing cells in the lineage, which indicates that they are all neurons (Fig. 5B, B'). Observations with both types of clones were confirmed by immunostaining for the neuronal marker 22C10. In the first type of clone, two GFP-positive cells express D-Pax2 (Fig. 5C') but none of the three cells expresses 22C10 (Fig. 5C'', C'''), which suggests they are support cells but not neurons (Fig. 5C-C'''). For the second type of clone, all GFP-positive cells express 22C10 but not D-Pax2,

which demonstrates that they are all neurons (Fig. 5D-D'''). These results suggest that the socket, the shaft, and the sheath cell are derived from one secondary precursor, the pIIa cell, while all neurons are derived from the other secondary precursor, the pIIb cell.

To determine the cell division patterns of the secondary precursors, pIIa and pIIb, clones were induced by heat shock in 5-6 hour-old embryos. Mainly four types of clones were observed (Table 2). One type consists of a single GFP-positive cell, the socket cell, as evident from its most dorsal location in this lineage (Fig. 6A, A'). Another type consists of the shaft and sheath cells, as both express D-Pax2 (Fig. 6B, B'). A third type consists of two cells that are bipolar neurons judged by their position in the lineage and the lack of D-Pax2 expression (Fig. 6C, C'). The last type of clone also consists of two cells, the third bipolar neuron and the md neuron, as the md neuron is always located at the most ventral position in the lineage (Fig. 6D, D'). As shown above, *Poxn* is expressed in one bipolar neuron during late stages of embryogenesis (Fig. 1D-F). We found that this *Poxn*-expressing neuron was in the same clone as the md neuron, as evident from the position of the latter (Fig. 6E, E') or the expression of the md neuron marker E7-2-36 (Fig. 6F, F').

The division pattern described above is different from any of the cell lineages reported previously (Bodmer et al., 1989; Brewster and Bodmer, 1995; Orgogozo et al., 2001). To confirm our observations, we analyzed the lineage, using a different Gal4 line, *D-Pax2-Gal4*^{Y283}. *D-Pax2-Gal4*^{Y283} is expressed in the SOPs and all their daughter cells of both p-es organs and m-es organs, which allows us to analyze both the kölbchen lineage as well as the m-es lineage in the same experiment. When the embryos were heat-shocked at 3-4 or 4-5 hours AEL, we observed the same types of clone in the kölbchen lineage as shown above (Fig. 5): all support cells are generated from the pIIa cell (Fig. 7A-A''), and all neurons are generated from the pIIb cell (Fig. 7B-B''). We also analyzed the lineage of hair 1 (h1), a typical representative of an m-es organ. We observed mainly two types of clones in the h1

lineage: one type consists of the socket and the shaft cell (Fig. 7C-C''), the other of the sheath cell, one bipolar neuron and the md neuron (Fig. 7D-D''). Hence, the cell division pattern observed in the h1 lineage is in accord with the previously reported model for the lineage of m-es organs (Orgogozo et al., 2001).

V. Cell lineage of kölbchen is altered in *Poxn* mutants

To test whether *Poxn* is necessary for the specification of the kölbchen lineage, it was analyzed in homozygous $Poxn^{\Delta M22}$ embryos. We first generated clones that included all cells produced by the lineage. Surprisingly, we observed two types of lineages in these embryos that produced either five (Fig. 8A, A') or six cells (Fig. 8B, B'). To exclude the possibility that this variation in cell number is an artefact of Gal4 that might induce an additional mitosis and to quantify both lineages, we analyzed the number of GFP-positive cells in $Poxn^{\Delta M22}$; $Poxn\text{-}CD8\text{:}GFP^{3\text{-}3}$ embryos. $Poxn\text{-}CD8\text{:}GFP^{3\text{-}3}$ is a transgene that expresses CD8:GFP under the direct control of the *Poxn* promoter and enhancer [the same as in the *Poxn* transgene *EvK* (Boll and Noll, 2002; W. Boll, unpublished]. In these embryos, again, we observed two types of kölbchen lineages that consisted of either five (Fig. 8C, C') or six cells (Fig. 8D, D'), which rules out that Gal4 causes the variation in cell number. We found that 65% (93 out of 144) of the mutated kölbchen consisted of five cells (Fig. 8C, C'), and 35% (51 out of 144) consisted of six cells (Fig. 8D, D').

To identify each individual cell of the kölbchen lineage of $Poxn^{\Delta M22}$ mutants, we used immunostaining and the cell markers mentioned above. When the lineage gave rise to five cells, these consisted of a socket cell that expresses Su(H) and is always located most dorsally (Fig. 9A, A'), a shaft cell that expresses D-Pax2, a sheath cell that is located more ventrally and expresses both D-Pax2 and Pros (Fig. 9B, B'), and two neurons (Fig. 9C, C', E, and E'). When the lineage produced six cells, these also consisted of three support cells (data

not shown), like those generated in the other lineage, but three neurons (Fig. 9D, D', F, and F'). Like in wild-type, the most ventrally located neuron of both lineages is an md neuron since it expresses the marker E7-2-36 (Fig. 9E-F'). These results suggest that in $Poxn^{\Delta M22}$ mutant embryos, the transformed kölbchen consist of all three support cells and one md neuron but a reduced number of one or two bipolar neurons.

To examine the cell division pattern of the kölbchen lineage in the absence of *Poxn*, $Poxn^{\Delta M22}$ embryos were subjected to heat shock at 4-5 hours AEL to improve the yield of clones derived from pIIa or pIIb cells (Table 3). We found mainly two types of GFP-positive clones. One type consisted of two cells, socket and shaft cells, that were identified by their position within the lineage and D-Pax2 staining, respectively (Fig. 10A-A''). The other type consisted of the D-Pax2 expressing sheath cell and two or three neurons (Fig. 10B-B''). The identities of cells in these two types of clones were further confirmed by Pros staining (Fig. 11). In the first type of clone (Fig. 11A-A'''), neither D-Pax2 (Fig. 11A) nor Pros (Fig. 11A') is expressed in the more dorsally located cell, which suggests that it is a socket cell. The other cell of this clone expresses only D-Pax2 (Fig. 11A) but not Pros (Fig. 11A'), which indicates that this cell is a shaft cell. In the second type of clone (Fig. 11B-B'''), one cell expresses both D-Pax2 (Fig. 11B) and Pros (Fig. 11B'), which shows that it is a sheath cell. The remaining cells of this clone are neurons, as evident from their more ventral localization and lack of expression of both D-Pax2 (Fig. 11B) and Pros (Fig. 11B'). We also analyzed lineages that consist of six cells and found the same types of clones (data not shown). This shows that, in the kölbchen lineage of $Poxn^{\Delta M22}$ mutants the socket and shaft cells are derived from the pIIa cell, while the pIIb cell generates the sheath cell and all neurons.

To determine the division pattern of the pIIb cell, embryos were heat-shocked at 5-6 hours AEL (Table 3). Mainly two types of clones were observed. One type consisted of two cells, the sheath cell and one bipolar neuron (Fig. 12A, A'). The other type consisted of

either only an md neuron, if the lineage consisted of five cells (Fig. 12B, B'), or a bipolar neuron and an md neuron if the lineage consisted of six cells (Fig. 12C, C').

Taken together, our results indicate that the kölbchen lineage is altered in homozygous *Poxn*$^{\Delta M22}$ embryos, which gives rise to fewer cells with a division pattern similar to that of the lineage of m-es organs.

VI. No evidence for apoptosis in the kölbchen lineage

Programmed cell death, or apoptosis, plays an essential role during metazoan development (for review, see Danial and Korsmeyer, 2004). In *Drosophila*, apoptosis has been reported in two cell lineages. In the microchaete lineage of adult mechanosensory bristles, it has been shown that the glial cell undergoes apoptosis (Fichelson and Gho, 2003), and in the md-solo lineage in the embryo, the pIIa and the pIIIb cells are eliminated by programmed cell death (Orgogozo, et al., 2002).

To test if the cells of the kölbchen lineage undergo apoptosis, we used an antibody that recognizes activated Caspase 3 (Casp 3), which is an apoptotic cell marker (Tewari et al., 1995). In the kölbchen lineage, cell division ceases by the end of stage 12. We assumed that apoptosis events, as visualized by Casp 3 immunostaining, should be observable between this time point and the end of cell differentiation (about stage 15). We collected embryos between late stage 12 and stage 15. In these embryos, cells derived from the kölbchen lineage were labeled by GFP. However, we did not observe activated Casp 3 in any GFP-expressing cell at any stage (Fig. 13A-B'', and data not shown), which suggests that there is no apoptosis in the kölbchen lineage in wild-type embryos. Meanwhile, we observed the staining of Casp 3 in neighboring cells (arrows in Fig. 13A, B) and in the CNS (data not shown), which indicated that the antibody staining was successful.

To confirm these observations, we undertook a complementary approach and inhibited cell death during the development of kölbchen by expressing the baculovirus anti-apoptotic protein P35 (Hay et al., 1994). When *UAS-p35* is expressed under the control of *Poxn-Gal4*^{ups1f} in the kölbchen lineage of wild-type embryos, the lineage still generates seven cells, and no extra CD2-positive cells were observed (Fig. 13C, C').

In *Poxn*$^{\Delta M22}$ embryos, the kölbchen lineage gives rise to a reduced number of cells (Fig. 8). To determine whether this reduction in cell number is due to cell death, we also analyzed apoptosis in the kölbchen lineage of homozygous *Poxn*$^{\Delta M22}$ embryos. As described above, there were two types of lineages in *Poxn*$^{\Delta M22}$ mutant embryos. We focused on clones that contain six cells at stages 13 or 14, as the mutated kölbchen lineage gives rise to at least five cells when analyzed at stage 16. When the embryos were stained with antibody against activated Casp3, again no co-expression of Casp 3 with CD2 was observed at any stage (Fig. 14A-B', and data not shown), while the antibody worked properly (Fig. 14A, B, arrows). We also expressed P35 in *Poxn*$^{\Delta M22}$ embryos under the control of *Poxn-Gal4*^{ups1f}. Again, we found two types of lineages, as described above (Fig. 8), that consisted of five or six cells, with a similar ratio as observed in embryos that do not express P35 (Fig. 14C-D').

These results suggest that none of the cells in the kölbchen lineage undergoes apoptosis in both wild-type and *Poxn*$^{\Delta M22}$ mutant embryos, and that the reduced number of cells in the kölbchen lineage of *Poxn*$^{\Delta M22}$ embryos is not caused by programmed cell death.

VII. Cell lineage of papilla 6

In a previous study, Brewster and Bodmer have proposed a model for the cell lineage of the p-es papilla p6 (Brewster and Bodmer, 1995). Their model exhibits a similar cell division pattern to the m-es lineage and is quite different from the kölbchen lineage just described. To

test whether the p-es lineages of kölbchen and p6 are indeed different, we decided to analyze the p6 lineage with the same method used to determine the kölbchen lineage.

It was had been proposed that the p6 lineage generates six cells, including three support cells, two bipolar neurons, and one md neuron (Brewster and Bodmer, 1995). However, when we generated clones in SOPs of p6 at 2-3 or 3-4 hours AEL, we always observed GFP expression in clones that consists of more than six cells (Fig. 15A-A’’’, Table 4). Besides the six cells that are derived from the p6 lineage, there are another three GFP-positive cells located at a more ventral and posterior position (Fig. 15A, arrow). The nine cells represent the entire lineage because heat shock was applied at a time long before SOPs were selected, as discussed above, and no CD2-positive cells were observed in this clone (Fig. 15A’). Moreover, these cells were derived from the same precursor cell because heat-shock conditions had been optimized to produce a single recombination event in a lineage and the vast majority of clones observed consisted of nine cells (hs at 2-3 hours AEL in Table 4).

The three cells have been described only briefly in an earlier publication where they were suggested to form an epidermal gland (Orgogozo and Schweisguth, 2004). The expression of GFP in these cells does not result from ectopic expression of *Poxn-Gal4* since Poxn is indeed expressed in all three cells, which can be clearly distinguished from the p6 cells at stage 13 (data not shown). Two of these three cells express D-Pax2 during late embryogenesis (Fig. 15C’’’), and none of them expresses the neuronal markers 22C10 or Elav (data not shown). When embryos were heat-shocked at 3-4 hours AEL, about half of the GFP-positive clones (Table 4) consisted of either the p6 cells (Fig. 15B-B’’’) or the presumptive epidermal gland cells (Fig. 15C-C’’’). In our studies, we focused only on the p6 lineage.

Our results show that the p6 lineage gives rise to six cells (Fig. 16A, A’), which is consistent with the previous report (Brewster and Bodmer, 1995). Using different cell markers to identify the cells derived from the p6 lineage, we found that the socket cell is

always located most dorsally, the shaft and the sheath cells express D-Pax2, and the three neurons are located more ventrally and express the neuronal marker 22C10 (Fig. 16A, A').

When heat shock was applied at 5-6 hours AEL to generate clones that consist of a subset of the p6 lineage (Table 4), we observed that the GFP-expressing clones consisted of either all support cells (Fig. 16B, B') or all neurons (Fig. 16C-D'), which indicates that all support cells are generated from one precursor, the pIIa cell, while all neurons are generated from the other precursor, the pIIb cell. Among the neurons, there is one md neuron (Fig. 16C-D'). Moreover, we also found that only one of the two bipolar neurons expressed BarH1 (Higashijima et al, 1992) (Fig. 16D, D'), which suggests that the two bipolar neurons acquire different cell fates.

We next analyzed the division patterns of the pIIa and pIIb cells by inducing clones at 6-7 hours AEL. We observed mainly four types of clones (Table 4). The first type consisted of the socket cell, as it is located most dorsally and does not express D-Pax2 or 22C10 (Fig. 17A, A'). The second type of clone consisted of the shaft and the sheath cell, as evident from D-Pax2 staining (Fig. 17B, B'). The third type of clone consisted of two bipolar neurons since they both express 22C10 but not the md neuron marker (Fig. 17C, C'). And the last type of clone was the md neuron (Fig. 17D, D').

VIII. One cell undergoes apoptosis in the p6 lineage

Our previous experiments indicate that apoptosis is not involved in the kölbchen lineage. However, we very often (43 out of 62) observed a GFP-positive cell adjacent to the p6 cells that was also stained for Casp 3 in embryos at stage 13 (Fig. 18A, A', asterisk). This cell is an extra cell, as the cells derived from the p6 lineage and the epidermal gland lineage are still present (Fig. 18A, A'). Moreover, when we inhibited apoptosis by the ectopic expression of P35, we also observed an extra cell close to the p6 cells (Fig. 18B, B', asterisk).

To determine whether the apoptotic cell is produced from the p6 lineage, we generated clones that consisted of the entire p6 lineage or a sub-lineage of it. Embryos carrying *UAS-p35* in addition to other transgenes used in previous lineage analyses were subjected to heat shock. When we analyzed GFP-expressing clones representing the entire p6 lineage, we found an extra cell in addition to the p6 cells (asterisk in Fig. 18C, C'). We did not observe this apoptotic cell in any clone that consisted of the epidermal gland cells (data not shown). These results suggest that the apoptotic cell is derived from the p6 lineage. In many cases (34 out of 55 clones), the apoptotic cell also expressed the neuronal marker Elav (asterisk in Fig. 18C, C'), which suggests that it acquired a neuronal fate. When clones were induced at 5-7 hours AEL, the apoptotic cell was found to be in the same GFP-expressing clones as the neurons (asterisk in Fig. 18D, D'). Furthermore, we observed clones that consisted of the md neuron and the apoptotic cell (asterisk in Fig. 18E, E'), which shows that the md neuron and apoptotic cell are derived from the same precursor.

Discussion

Late expression of *Poxn* during the development of kölbchen

The expression pattern of *Poxn* in cells of the embryonic PNS has been described in a previous study (Dambly-Chaudière et al., 1992), which showed that *Poxn* was expressed in the SOPs and their daughter cells forming p-es organs in the thoracic and abdominal segments and stated that the expression of *Poxn* declined by the time when those cells began to differentiate. However, we observed the continuous expression of *Poxn* in a single bipolar neuron innervating each kölbchen upon the completion of germband retraction, and this expression is still detectable during later developmental stages (Fig. 1D-F) until the end of embryogenesis.

One possible function of this late expression of *Poxn* is to prevent this neuron from apoptosis (see below). In addition, *Poxn* might be required for the differentiation of this neuron. Kölbchen are innervated by three bipolar neurons. Currently, the biological functions of these neurons remain unclear. However, it has been suggested that kölbchen are involved in chemosensory detection: for instance, the *enhancer-Gal4* transgene - in which *Gal4* is activated by the enhancer of one of the candidate gustatory receptors in *Drosophila*, Gr2B1 - has been shown to be expressed in one of the neurons innervating the ventral kölbchen, which suggests that kölbchen may play a role in taste perception of larvae (Scott et al., 2001). This late expression of *Poxn* in a single neuron might lead to cell-specific expression of some chemosensory receptors, which allow this neuron to respond to certain environmental stimuli and process the sensory information.

Our observation of the late expression of *Poxn* in the PNS during embryogenesis seems at odds with the previous report (Dambly-Chaudière et al., 1992). A possible explanation is that

the expression of *Poxn* in the epidermis during late embryogenesis (Fig. 1E, F) interfered with the observation in that study.

Cell lineage of kölbchen

The cell lineage of kölbchen has not been analyzed to date. In this study, we describe a cell lineage for kölbchen (Fig. 19A) that is not only different from an earlier model proposed for larval p-es organs (Jan and Jan, 1993; Brewster an Bodmer, 1995), but also distinct from any of the previously reported sensory organ cell lineages.

The kölbchen lineage gives rise to seven cells through three rounds of cell division (Fig. 19A). The SOP, or pI cell, divides first to generate two secondary precursor cells, pIIa and pIIb. The pIIa cell in turn divides to give rise to the socket cell and the tertiary precursor pIIIa, which divides once more to produce the shaft and the sheath cell. The pIIb cell gives rise to two tertiary precursors, pIIIb1 and pIIIb2, each of which will divide again to produce two neurons, pIIIb1 two bipolar neurons, and pIIIb2 an md neuron and a third bipolar neuron, which is the neuron that continues to express *Poxn* during late embryogenesis (Fig. 19A).

The kölbchen lineage described in our study exhibits significant differences to the m-es lineage (compare Fig. 19A with Fig. 19B). The kölbchen lineage gives rise to more neurons compared to the m-es lineage but, most strikingly and in contrast to the m-es lineage, all support cells, i.e., socket, shaft, as well as sheath cell, are derived from the pIIa cell. The pIIb cell, however, is the precursor of all neurons (Fig. 19A). In the m-es lineage, the socket and shaft cell are derived from the pIIa cell, while the sheath cell is generated from the pIIb cell, which gives also rise to a bipolar neuron and an md neuron (Fig. 19B).

The division pattern of the kölbchen lineage suggests that the shaft cell, sheath cell, and all neurons are generated simultaneously during embryogenesis (Fig. 19A). Although our method could not determine the order of cell divisions among the tertiary precursors pIIIa,

pIIIb1, and pIIIb2, the similar number of each type of clones scored during our analysis (5-6 hours AEL in Table 2) suggests that all three cells divide within a very short time interval. Given the limited time for development during embryogenesis, one advantage of such a division pattern is to permit all support cells and neurons a similar time period for differentiation and subsequent formation of the sensory organ. If the cell division pattern of the kölbchen lineage was similar to that of the m-es lineage, it would require at least one additional round of cell divisions in the pIIb cell lineage until the sheath cell and all neurons are generated. Meanwhile the socket and the shaft cell have already been produced from the pIIa cell but might not be able to differentiate properly in the absence of the sheath cell and neurons. Clearly, such a division pattern would delay differentiation of the cells and thus might affect the formation of kölbchen.

Our model also suggests that most cell divisions in the kölbchen lineage are asymmetric cell divisions, as the daughter cells acquire different cell fates (indicated by different colors in Fig. 19A). However, there is one cell division, the pIIIb1 division, that might be symmetric, as it generates two bipolar neurons (Fig. 19A) and no cell-specific marker was found to distinguish them. Although it was reported that *BarH1* is expressed in one neuron associated with kölbchen (Higashijima et al, 1992), when embryos were stained with anti-BarH1 antibody, no expression of *BarH1* was detected in any of the neurons innervating the kölbchen, whereas *BarH1* expression was observed in all three support cells (data not shown). However, there might be an alternative way to distinguish these two neurons based on their morphologies. It has been shown that only two of the three bipolar neurons have their dendrites project into the lumen of the shaft, while the dendrite of the third bipolar neuron terminates beneath the base of the kölbchen (Hartenstein, 1988). Labeling the neurons with a membrane-bound marker CD8:GFP expressed under the control of a pan-neuronal Gal4 driver like *Elav-Gal4*, we might be able to observe the dendrite projections of

all three bipolar neurons innervating kölbchen. Since *Poxn-Gal4* is expressed only in the bipolar neuron derived from pIIIb2, comparison of the dendrite projections of the other two neurons might permit us to determine whether they acquire different cell fates.

Asymmetric cell divisions have been intensively studied in the lineage of adult mechanosensory bristles and the division of neuroblasts in the CNS (Betschinger and Knoblich, 2004). During asymmetric cell division, cell fate determinants, like Numb, localize asymmetrically during mitosis and later segregate into only one of the two daughter cells, which leads to the activation of the Notch signaling pathway in only one of them and thus results in different cell fates (Betschinger and Knoblich, 2004). It would be interesting to analyze in future experiments how the proteins that determine cell fates are localized during the cell divisions and how Notch signaling is regulated in the kölbchen lineage.

Poxn also plays an essential role during development of the chemosensory bristles on legs, wings, and labellum of adult *Drosophila* (Awasaki and Kimura, 1997; Boll and Noll, 2002). Therefore, from an evolutionary point of view, the kölbchen lineage described in this study might be similar to that of the poly-innervated chemosensory bristles of adult flies. The cell lineage of the taste bristles on the labellum has been analyzed in a previous report by labeling replicating cells with 5-bromo-2-deoxyuridine (BrdU) (Ray et al., 1993). However, the model proposed in that study is similar to the m-es lineage: the pIIa cell is the precursor of the socket and shaft cell, and the sheath cell and all neurons are derived from the pIIb cell (Ray et al., 1993). Given the technical limitation of BrdU incorporation and the lack of using cell-specific markers in that study, the cell lineage of taste bristles on the labellum might need to be re-investigated.

Altered cell lineages of kölbchen in *Poxn* mutants

In homozygous $Poxn^{\Delta M22}$ larvae, kölbchen are transformed into papilla-like sensory organs, in agreement with previous observations of a strong hypomorphic *Poxn* mutant, $Poxn^{70}$, or of a large deficiency, *Df(2R)WMG*, uncovering *Poxn* (Awasaki and Kimura, 2001; Dambly-Chaudière et al., 1992). Our analysis of the kölbchen lineage in $Poxn^{\Delta M22}$ mutants shows that this transformation does not only result from changes of the external and internal structures of the kölbchen but, most important, from a transformation of the cell lineage (Fig. 19C, D).

First, in homozygous $Poxn^{\Delta M22}$ embryos, the kölbchen lineage gives rise to fewer cells that include all support cells and a reduced number of neurons (Fig. 19C, D). Second, there are two alternative lineages in $Poxn^{\Delta M22}$ mutants: about two thirds of the papilla-like sensory organs consist of five cells (Fig. 19C), while one third consists of six cells, as they include an additional bipolar neuron (Fig. 19D). Third, the cell division pattern is changed: in $Poxn^{\Delta M22}$ mutants, the pIIa cell becomes the precursor of the socket and the shaft cell, while the pIIb cell gives rise to the sheath cell and neurons (Fig. 19C, D). When the lineage produces five cells, the division pattern is transformed exactly into that of the wild-type m-es lineage (compare Fig. 19C with Fig. 19B), which suggests a complete transformation of the kölbchen into an m-es organ. When the lineage gives rise to six cells, the division pattern is similar to that of the m-es lineage, except that the pIIIb2 cell divides to produce an additional bipolar neuron and the md neuron (compare Fig. 19D with Fig. 19B).

It is surprising that not all kölbchen are transformed completely into m-es organs in the absence of *Poxn*. In a previous study, Awasaki and Kimura analyzed the internal structures of kölbchen in *Poxn* mutants and reported that the number of bipolar neurons was reduced to one (Awasaki and Kimura, 2001). However, we noted that their conclusion was based on the observation by transmission light microscopy of 22C10-labeled neurons that are clustered and hence difficult to discern. Therefore, an additional neuron could be easily missed in their

study. By contrast, we used clonal analysis to label the kölbchen cells by both membrane and nuclear markers, and we examined the clones by confocal microscopy, which allowed us to analyze the internal structures of kölbchen in *Poxn*$^{\Delta M22}$ mutants in three dimensions.

The incomplete transformation of kölbchen to m-es organs observed in *Poxn*$^{\Delta M22}$ embryos is also consistent with the phenotype observed in *Poxn*$^{\Delta M22}$ adult flies. Although in *Poxn*$^{\Delta M22}$ mutant flies, the chemosensory bristles on legs and wings are transformed to mechanosensory-like bristles, one or two chemosensory neurons are still present in about 10% of these transformed bristles on the male prothoracic leg, as visualized by the expression of GFP driven by *Poxn-Gal4* (W. Boll, unpublished data). Since *Poxn*$^{\Delta M22}$ is a null allele (Boll and Noll, 2002), these incomplete transformation phenotypes might be explained as follows. As the lineages of pIIa and pIIb are always changed and the lineage of pIIb is variable, we assume that a factor, e.g. D-Pax2, is required in pIIb cells, in partial redundancy with Poxn, to generate the wild-type lineage of pIIb. In the absence of Poxn, this factor suffices only in a third of the pIIb cells to induce its pIIIb2 daughter to undergo another cell division that generates an additional bipolar neuron. Therefore, it would be interesting to analyze the lineage of kölbchen in double mutants of *Poxn* and *D-Pax2* to see whether its lineage is completely transformed to that of an m-es organ. All these observations suggest that *Poxn* does not act simply as a binary switch in the determination of p-es organs in both larvae and adults.

Cell lineage of p6

We also analyzed the lineage of another larval p-es organ, papilla p6, to test whether the characteristics of the lineage described above is specific for kölbchen. Our results clearly demonstrate that this is not the case, as the p6 lineage is very similar to the kölbchen lineage. The p6 lineage gives rise to six cells, consisting of three support cells, two bipolar neurons,

and one md neuron (Fig. 20B). As for kölbchen, all support cells are derived from the pIIa cell while all neurons are generated from the pIIb cell (Fig. 20B).

The p6 lineage also differs from the kölbchen lineage in several aspects: (i) the SOP cell of p6 and the precursor of a presumptive epidermal gland are derived from the same precursor expressing *Poxn* (Fig. 20A); (ii) the p6 lineage gives rise to only two bipolar neurons, whereas three bipolar neurons are produced in the kölbchen lineage; (iii) none of the two bipolar neurons in the p6 lineage expresses *Poxn* during late embryonic stages but one of them expresses *BarH1* (Fig. 16D); and (iv) one cell undergoes apoptosis in the p6 lineage (see below).

The lineage of p6 has been proposed in a previous study where its cell division pattern was suggested to be similar to that of the m-es lineage (Fig. 20C; Brewster and Bodmer, 1995). The differences between our model and that previously proposed are significant (compare Fig. 20B with Fig. 20C). In the previous study, Brewster and Bodmer used an *act>Draf>nuclacZ* transgene to label the induced clones and staining for 22C10 to identify them in the PNS. Since the transgene they used is expressed ubiquitously, they very often observed labeling of adjacent ectodermal cells with LacZ (Brewster and Bodmer, 1995). These ectodermal clones might interfere with their analysis of the PNS clones. Moreover, the number for clones consisting of the sheath cell and all neurons was far less compared to other types of clones (see Table 1 in Brewster and Bodmer, 1995), and no picture of such a clone was shown.

The lineage of p6 in *Poxn* mutants generates five cells. Analysis of these mutant p6 organs is complicated by the fact that all five cells lie on top of each other. Preliminary studies indicate that two D-Pax2 expressing cells are present, presumably the shaft and the sheath cell. Assuming that all support cells are maintained in the mutant, as they are in mutant kölbchen, one of the neurons is not formed in mutant p6 organs. We speculate that

the cell division pattern is also altered, like in mutant kölbchen, to that of m-es organs. However, this remains to be established by future experiments.

Apoptosis in the p-es lineages

We did not find any programmed cell death in the kölbchen lineage, either by staining the embryos with an apoptotic cell marker (Fig. 13A, B) or by expression of an anti-apoptosis protein in the lineage (Fig. 13C). In addition, no apoptosis was observed in the kölbchen lineage of *Poxn* mutants even though the lineage produced fewer cells than in wild-type embryos (Fig. 14), which suggests that it results from a reduction of cell divisions.

However, we found that one cell in the p6 lineage undergoes apoptosis immediately after the last cell division (Fig. 18A). When apoptosis is inhibited in the p-es lineage, an extra cell is generated in the p6 lineage (Fig. 18B). In many cases (34 out of 55 clones), the extra cell expresses the neuronal marker Elav (Fig. 18C), which suggests that it acquires a neuronal fate. However, we did not see any dendritic outgrowth from this cell, which indicates that this surviving cell cannot properly differentiate into a functional bipolar neuron.

The apoptosis observed in the p6 lineage might be induced by the absence of the late *Poxn* expression since the corresponding cell derived from the pIIIb2 cell in the kölbchen lineage continues to express *Poxn* throughout late embryogenesis (compare Fig. 19A with Fig. 20B). One of the *Poxn* partial rescue transgenes, *Poxn-PK*, is expressed in the SOPs and their daughter cells, but not in the single bipolar neurons of kölbchen during late embryogenesis. Thus, whether *Poxn* plays a role to prevent apoptosis in these neurons could be tested by analyzing cell death in the kölbchen lineage in $Poxn^{\Delta M22}$ *Poxn-PK* embryos.

Apoptosis has been reported in other cell lineages in *Drosophila*. In the microchaete lineage of adult *Drosophila*, it has been shown that the glial cell undergoes apoptosis (Fichelson and Gho, 2003). Moreover, in the md-solo lineage in the embryo, both the pIIa

and pIIIb cells are eliminated by apoptosis (Orgogozo, et al., 2002). The apoptosis in the p6 lineage reported here is, to our knowledge, the first example in the p-es lineages of larvae and adult flies. Thus, apoptosis seems to be more ubiquitously used during the PNS development and plays an evolutionarily conserved role in the specification of sensory organ lineages.

In the md-solo lineage, Numb is necessary and sufficient to prevent apoptosis (Orgogozo, et al., 2002). It would be interesting to test whether Numb as well as other cell fate determinants play the same role in the p6 lineage. Moreover, Technau and colleagues recently reported that the Hox genes *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*) regulate segment-specific apoptosis of differentiated motoneurons in the ventral nerve cord of *Drosophila* embryos (Rogulja-Ortmann, et al., 2008). *Ubx* is required to activate segment-specific apoptosis in these cells, while their survival depends on *Antp*. Whether the apoptosis observed here in the p6 lineage is regulated through the same mechanism by Hox genes needs to be further investigated.

The role of *Poxn* during development of larval p-es organs

We have shown that the larval p-es organs are produced through novel cell lineages. Our loss-of-function study clearly shows that *Poxn* plays an essential role in the specification of the p-es lineages. The lineage analyses and the temporal and spatial expression of *Poxn* described in this study suggest several functions of *Poxn* during development of p-es organs. First, *Poxn* plays a role in regulating the number of cell division, as both the number of cells and the number of cell division in the kölbchen lineage is reduced in *Poxn* mutants (Fig. 19A, C, D). Second, *Poxn* also controls the cell division pattern in the p-es lineages, as the p-es lineages are transformed into m-es-like lineages in the absence of *Poxn* (Fig. 19C, D; for p6, see above). However, we do not know whether these functions of *Poxn* are required only in the SOP cell during the first cell division or whether the early expression of *Poxn* in the SOP

cell produces its effect in later cell divisions. To answer this question, we need to be able to manipulate the expression of *Poxn* in the secondary and tertiary precursor cells during cell division, for example by inducing *Poxn* mutant clones in the secondary and tertiary precursors with the use of the MARCM system (Lee and Luo, 1999). Unfortunately, it is impossible to perform such a manipulation in embryos because of the rapid progression of cell divisions during sensory organ development compared to the perdurance of Gal80 and Gal4 used in MARCM analysis. *Poxn* is also required for the formation of the chemosensory bristles in the adult *Drosophila*. It might be possible to manipulate the expression of *Poxn* in imaginal discs during late larval and early pupal stages, as the time interval between the division of the SOP and that of secondary precursor cells is much longer than during embryonic development. Third, the late expression of *Poxn* in one of the neurons innervating kölbchen indicates a late function of *Poxn* in these bipolar neurons. *Poxn* might be required to prevent apoptosis in this neuron during the development of kölbchen. Last, *Poxn* might also play a role in the differentiation of these bipolar neurons, as discussed above.

The levels of Poxn protein, as detected by antibody staining, do not indicate an asymmetric localization of Poxn during cell divisions in the p-es lineages. How does *Poxn* control the p-es lineages? One possibility is that, as a transcription factor, Poxn controls the cell division pattern of p-es lineages by regulating the expression of its target genes. One type of its targets might be genes encoding proteins required for asymmetric cell division, either cell fate determinants or proteins involved in cell-cell communication. Another type of its targets might encode proteins involved in the regulation of the cell cycle and cell proliferation, which has a direct effect on cell divisions. Another possibility is that *Poxn* might cooperate with other transcription regulators, whose expression or activity is directly controlled by asymmetric cell division.

We have shown that *Poxn* is required for the specification of the p-es lineages. It has been shown previously that ectopic kölbchen could form upon ectopic expression of *Poxn* (Dambly-Chaudière et al., 1992). However, the positions of these ectopic kölbchen did not seem to correspond to, and hence form upon, the transformation of m-es organs. To test whether *Poxn* is also sufficient to transform the m-es lineage into the p-es lineage, we ectopically expressed *Poxn* in the SOP cells of larval m-es organs using three different Gal4 drivers, *D-Pax2-Gal4*, *achaete-Gal4*, and *daughterless-Gal4*. However, we did not observe a transformation of m-es organs to p-es organs in larvae when *Poxn* is ectopically expressed during embryogenesis under the control of *D-Pax2-Gal4* and *achaete-Gal4*, whereas no larvae survived to the first instar using *daughterless-Gal4* (data not shown). One possibility is that Poxn is not sufficient to determine the p-es lineage by itself, which again suggests that Poxn does not act as a binary switch in the determination of p-es organs. An alternative explanation is that, in these ectopic expression experiments, *Poxn* was not expressed in the correct temporal fashion – its expression was either delayed (when using *D-Pax2-Gal4*) or it was expressed too early (when using *achaete-Gal4*) during the development of the m-es organs. Hence, to perform such an analysis, it would be crucial to ectopically express *Poxn* in the SOP cells and all their progenies of m-es organs in the same temporal and spatial pattern as *Poxn* is expressed in the p-es lineage. For instance, by tests using a transgene that expresses *Poxn* directly under the control of the *D-Pax2* enhancer and promoter, as *D-Pax2* is expressed in all precursors of the m-es lineage.

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Figures and Tables

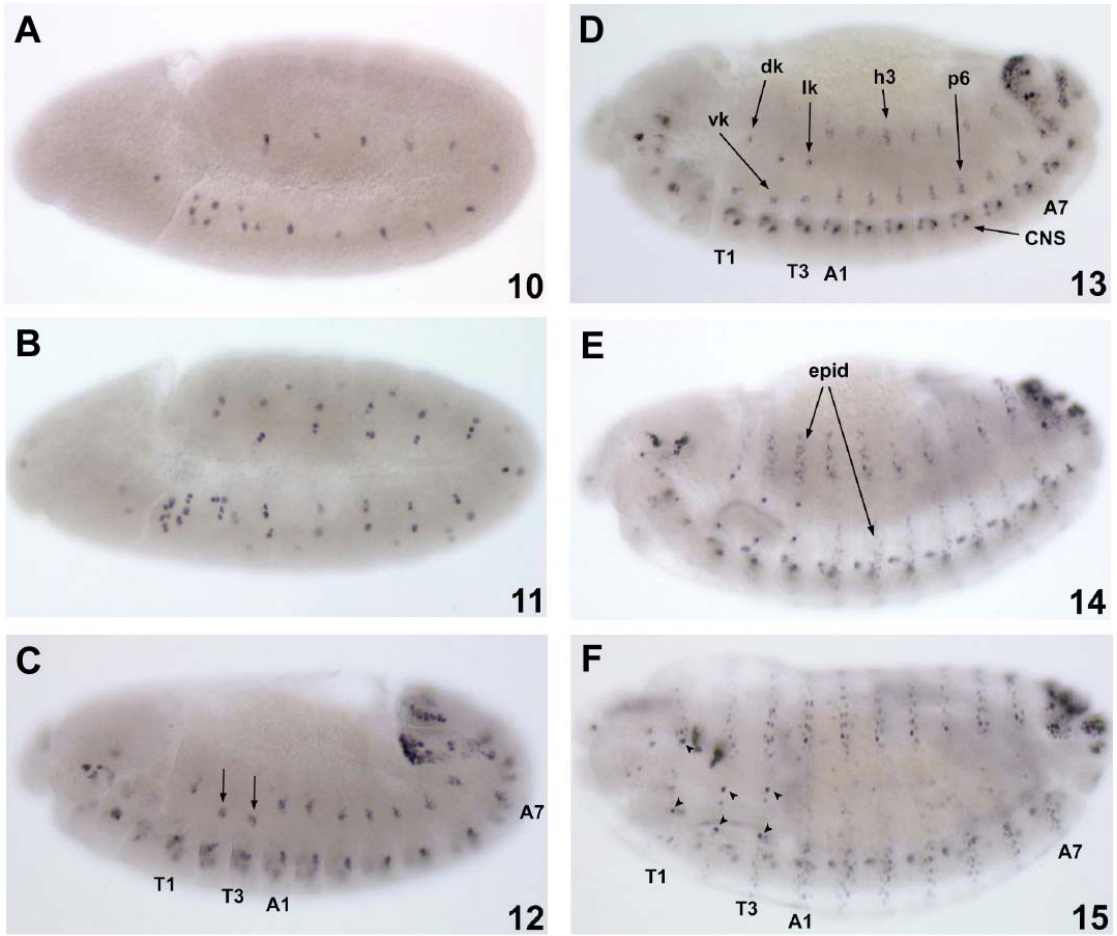

Fig. 1. Expression of *Poxn* during embryogenesis (courtesy of Werner Boll). Whole-mount wild-type embryos were stained with purified anti-Poxn antiserum. Lateral views of entire embryos at late stage 10 (A), stage 11 (B), stage 12 (C), stage 13 (D), stage 14 (E), and stage 15 (F) are shown. Note that the dorsal *Poxn*-expressing cells in T2 and T3 are located more ventrally at stage 12 (arrows in C). Arrowheads in (F) indicate the bipolar neurons innervating the kölbchen. Embryos are oriented with their anterior to the left and dorsal side up. Abbreviations: T, thoracic segments; A, abdominal segments; dk, dorsal kölbchen; lk, lateral kölbchen; vk, ventral kölbchen; h3, hair 3; p6, papilla 6; CNS, central nervous system; epid, epidermis.

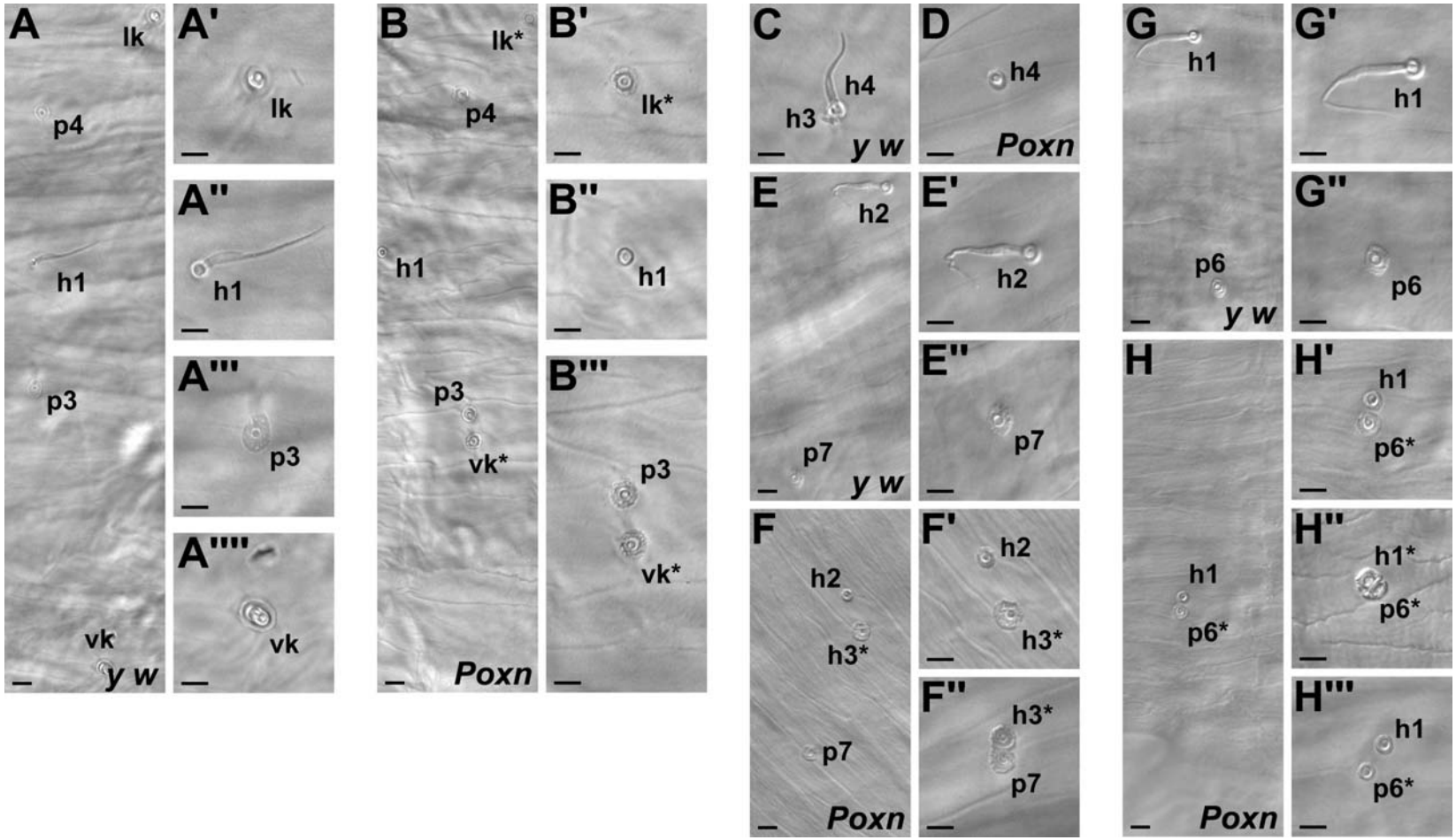

Fig. 2. Impaired external sensory organs in homozygous *Poxn*$^{\Delta M22}$ larvae. Lateral region of the third thoracic segment of third instar *y w* (A) and *y w; Poxn*$^{\Delta M22}$ larvae (B) with enlarged views of external sensory organs (A'-A'''' and B'-B''') are shown. Note that in *Poxn*$^{\Delta M22}$ larvae, kölbchen are transformed into papilla-like organs and the ventral kölbchen are located more dorsally close to papilla 3 (cf. B with A), and that hairs have degenerated shafts (cf. B'' with A''). Sensory organs in the dorsal (C, D), dorsolateral (E, F), and ventrolateral (G, H) regions of abdominal segments of third instar *y w* (C, E, G) and *y w; Poxn*$^{\Delta M22}$ larvae (D, F, H) are shown with corresponding enlarged views to the right of panels E-H. Anterior is to the left and dorsal side up. Scale bars are 5 μm.

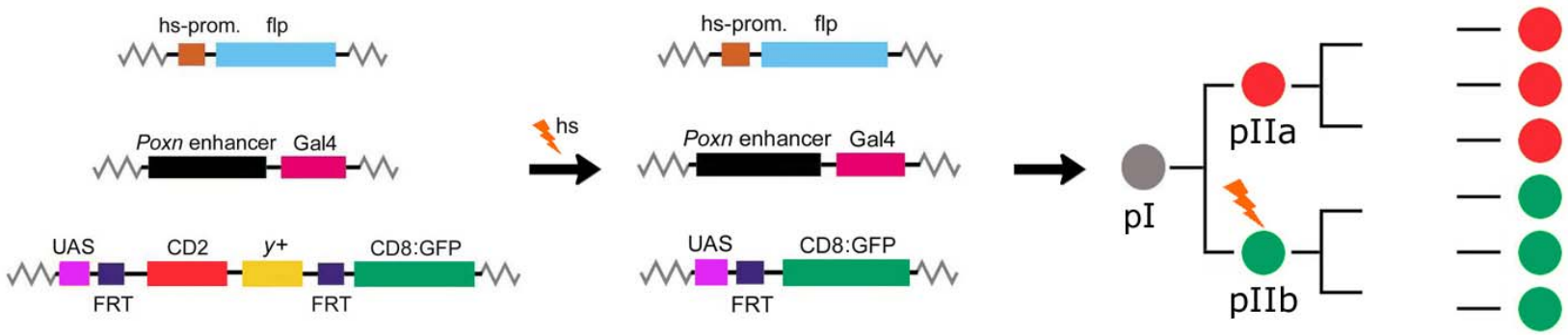

Fig. 3. Clonal analysis by *flp*-mediated site-specific recombination. Heat shock (hs) induces the heat-shock promoter (hs-prom.) that controls transcription of yeast *flp* recombinase (flp). This flipase mediates recombination between two FRT sites during cell divisions, thus generating clones that express CD8:GFP (green) under the control of Gal4 regulated by the *Poxn* enhancer active in the cell lineages of larval p-es organs. Cells of this lineage in which no recombination was induced express CD2 (red). Transgenes are not drawn to scale.

Temperature and duration of heat shock	Average number of clones	
	Thoracic	Abdominal
30°C, 30 min.	0.53	1.36
32°C, 25 min.	0.89	1.94
32°C, 30 min.	0.86	2.40
34°C, 15 min.	1.35	3.39
34°C, 30 min.	1.97	6.27

Table 1. Average number of GFP-expressing clones per embryo in the thoracic segments and abdominal segments A1-A7 after induction of *flp* by different heat-shock conditions. For each test, several hundreds of embryos were analyzed.

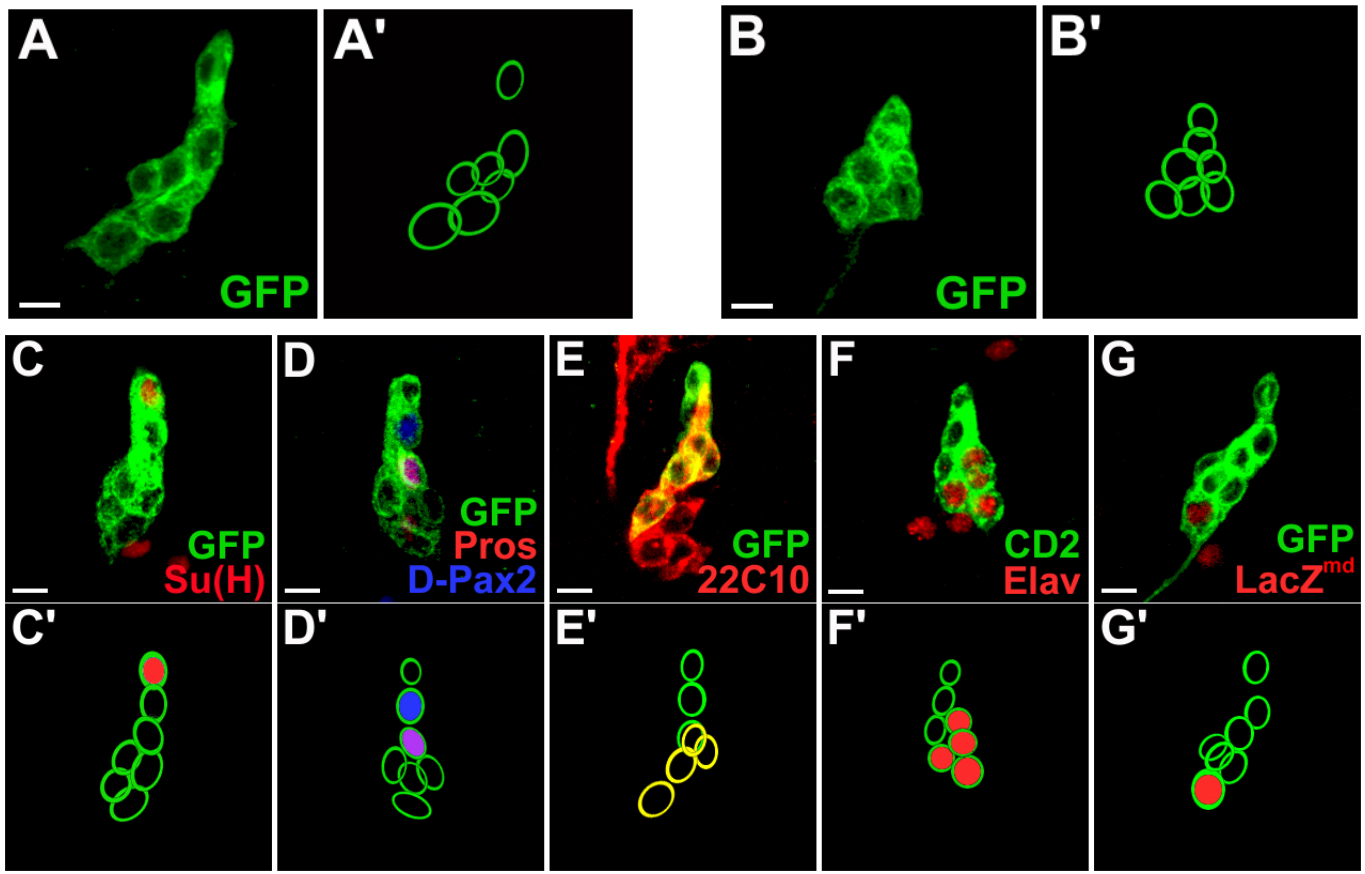

Fig. 4. The kölbchen lineage gives rise to seven cells**. (A-G)** Wild-type embryos were heat-shocked for 30 minutes at 2-3 hours AEL (except for F, which shows a clone of an embryo that was not heat-shocked) and immunostained with the indicated antibodies. GFP-expressing clones induced in lateral (A) or ventral (B) kölbchen contain seven cells, including a socket cell that expresses Su(H) (C), a shaft cell that expresses D-Pax2 and a sheath cell that expresses both D-Pax2 and Pros (D), and four neurons (E, F), among which the most ventrally localized neuron expresses the md neuron marker LacZ (G). **(A'-G')** Schematic representations of the clones in the corresponding panels A-G. Scale bars are 5 µm.

Genotypes: (A-F) *y w hs-flp*; *UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *Poxn-Gal4*^{ups1f}*/TM6B*; (G) *y w hs-flp*; *E7-2-36 UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *Poxn-Gal4*^{ups1f}*/TM6B*.

type of clones / hs at [hours AEL]	all cells	three support cells	four neurons	socket	shaft & sheath	two bipolar neurons	one bipolar neuron & an md	others
2 – 3	83	7	10	0	0	0	0	0
3 – 4	42	24	31	0	0	0	0	0
4 – 5	7	44	53	2	3	4	1	1
5 – 6	0	10	13	10	41	33	30	6

Table 2. Numbers and types of clones scored in the kölbchen lineage when wild-type embryos were heat-shocked for 30 minutes at 32°C at different developmental times (time intervals correspond to development at 25°C).

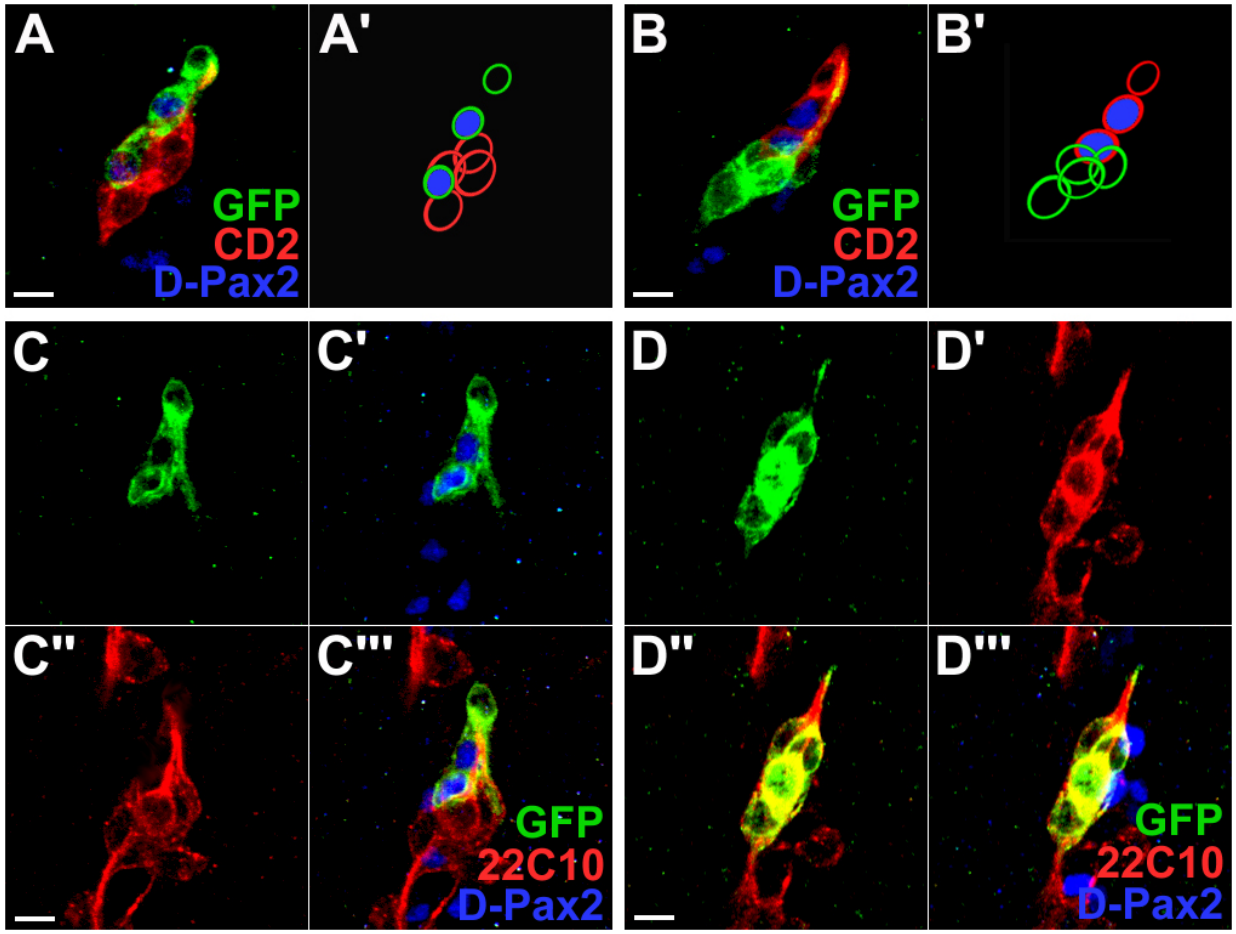

Fig. 5. Representative clones in the kölbchen lineage, induced at 3-4 or 4-5 hours AEL. Wild-type embryos were heat-shocked for 30 minutes at 3-4 or 4-5 hours AEL and immunostained with the indicated antibodies. Mainly two types of GFP-expressing clones in the kölbchen lineage were observed. The first type of clone consists of the three support cells (A, C), as evident from their position in the lineage (A), the expression of D-Pax2 (A, C'), and the lack of 22C10 expression (C'', C'''). The second type of clone consists of four neurons (B, D), which are located more ventrally in the lineage (B), all of which express the neuronal marker 22C10 (D', D'') but not D-Pax2 (B, D'''). **(A', B')** Schematic representations of the clones shown in A and B. Scale bars are 5 µm.
Genotypes: *y w hs-flp*; *UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *Poxn-Gal4*^{ups1f}*/TM6B*.

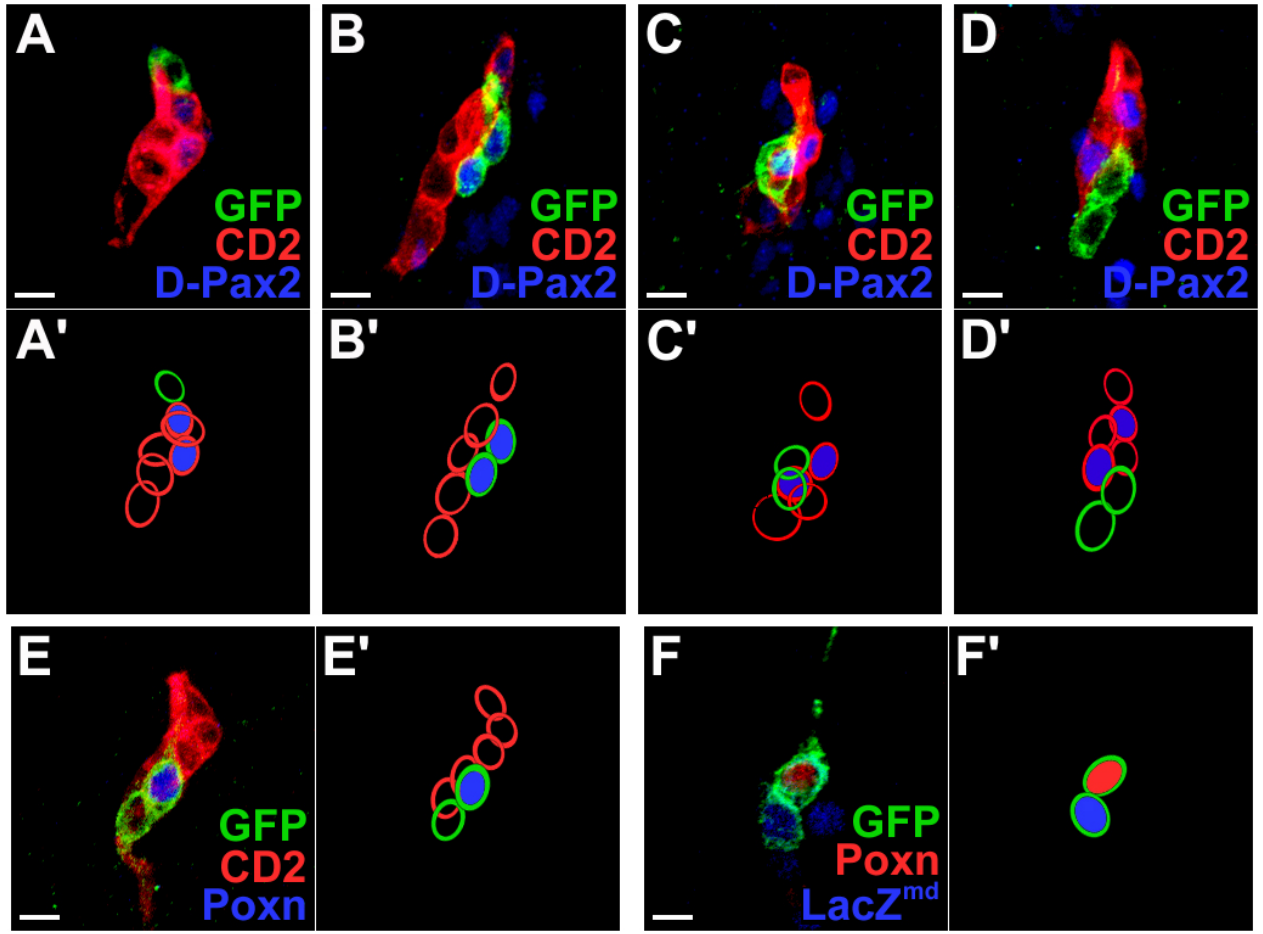

Fig. 6. Representative clones in the kölbchen lineage, induced at 5-6 hours AEL. Wild-type embryos were heat-shocked for 30 minutes at 5-6 hours AEL and immunostained with the indicated antibodies. **(A-F)** GFP-expressing clones in the kölbchen lineage consist of the socket cell (A), the shaft and sheath cell (B), two bipolar neurons (C), and one bipolar neuron and the md neuron (D-F). The bipolar neuron that expresses *Poxn* during late embryogenesis was observed in the same clone as the md neuron (E, F). **(A'-F')** Schematic representations of the clones in the corresponding panels A-F. Scale bars are 5 µm.

Genotypes: (A-E) *y w hs-flp*; *UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *Poxn-Gal4*^{ups1f}*/TM6B*; (F) *y w hs-flp*; *E7-2-36 UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *Poxn-Gal4*^{ups1f}*/TM6B*.

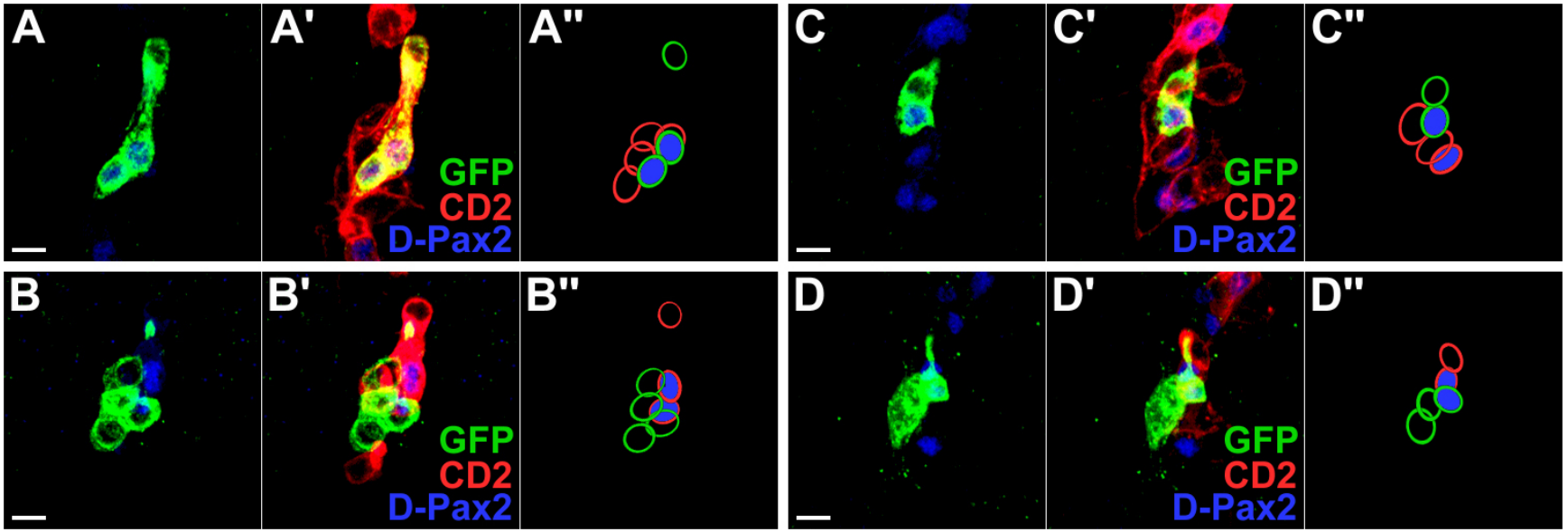

Fig. 7. GFP-expressing clones labeled by the use of *D-Pax2-Gal4*. Wild-type embryos were heat-shocked at 3-4 or 4-5 hours AEL and immunostained with the indicated antibodies. Representative GFP-expressing clones, induced either in the kölbchen lineage (A-B'') or in the m-es hair 1 lineage (C-D''), are shown. Two types of clones were observed in the kölbchen lineage that consist of either all support cells (A, A') or all neurons (B, B'). In the hair 1 lineage, also two types of clones were observed: one type of clone consists of the socket and the shaft cell (C, C'), and the other consists of the sheath cell, one bipolar neuron, and the md neuron (D, D'). **(A''-D'')** Schematic representations of the clones in the corresponding panels A'-D'. Scale bars are 5 µm.
Genotypes: *y w hs-flp*; *UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *D-Pax2-Gal4*^{Y283}.

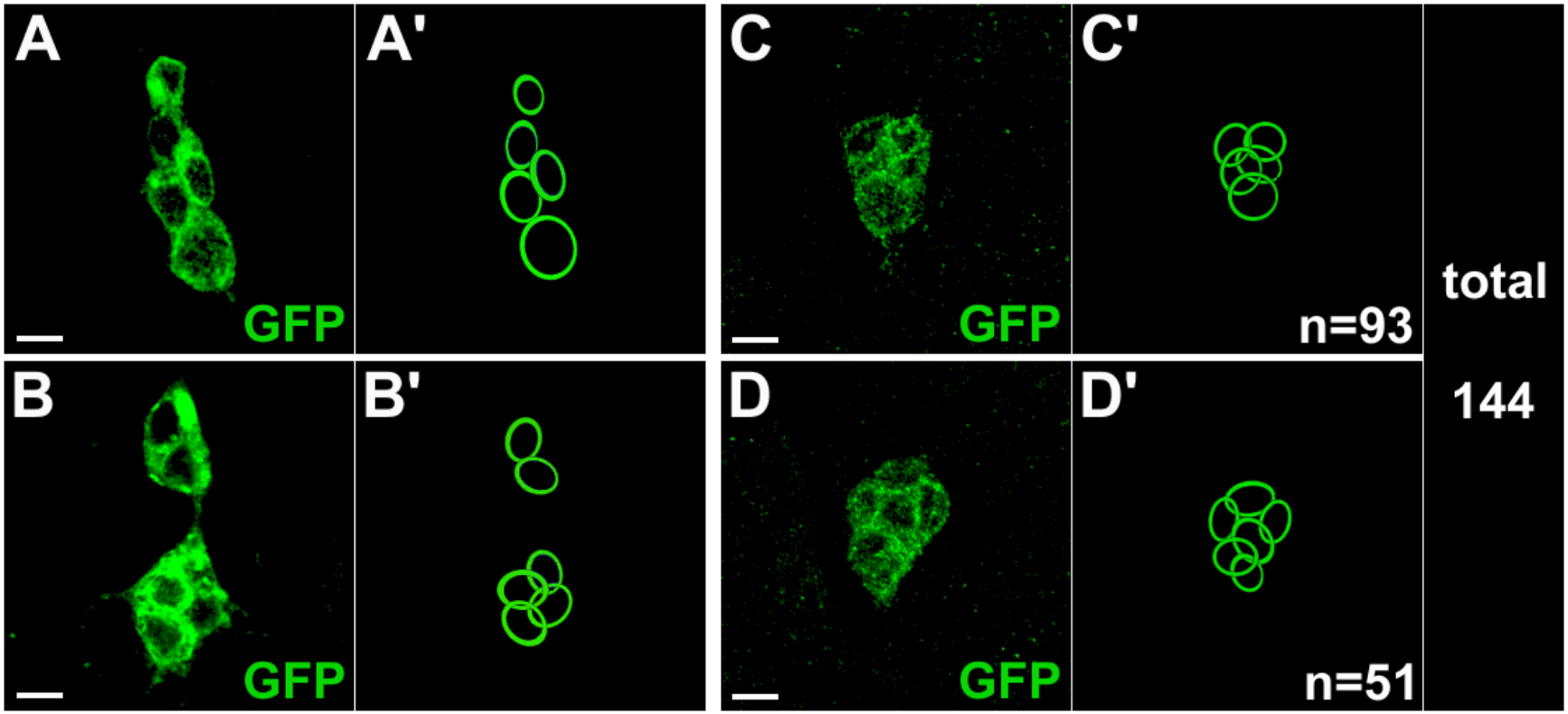

Fig. 8. Two alternative lineages of kölbchen in *Poxn*$^{\Delta M22}$ embryos. **(A, B)** Homozygous *Poxn*$^{\Delta M22}$ embryos were heat-shocked at 3-4 hours AEL and representative GFP-expressing clones that consist of the entire lineage of kölbchen are shown. Two alternative lineages were observed that gave rise to either five (A) or six (B) cells. **(C, D)** Stage 13 embryos of *w; Poxn*$^{\Delta M22}$*; Poxn-CD8:GFP* were immunostained for GFP. Two types of GFP-expressing cells were observed: about 65% (93 out of 144) consist of five cells (C) and 35% (51 out of 144) consist of six cells (D). **(A'-D')** Schematic representations of the clones in the corresponding panels A-D. Scale bars are 5 µm.
Genotypes: (A, B) *y w hs-flp*; *Poxn*$^{\Delta M22}$ *UAS>CD2, y*$^{+}$*>CD8:GFP*; *Poxn-Gal4*$^{ups\,1f}$*/TM6B*; (C, D) *w*; *Poxn*$^{\Delta M22}$; *Poxn-CD8:GFP*$^{3\text{-}3}$.

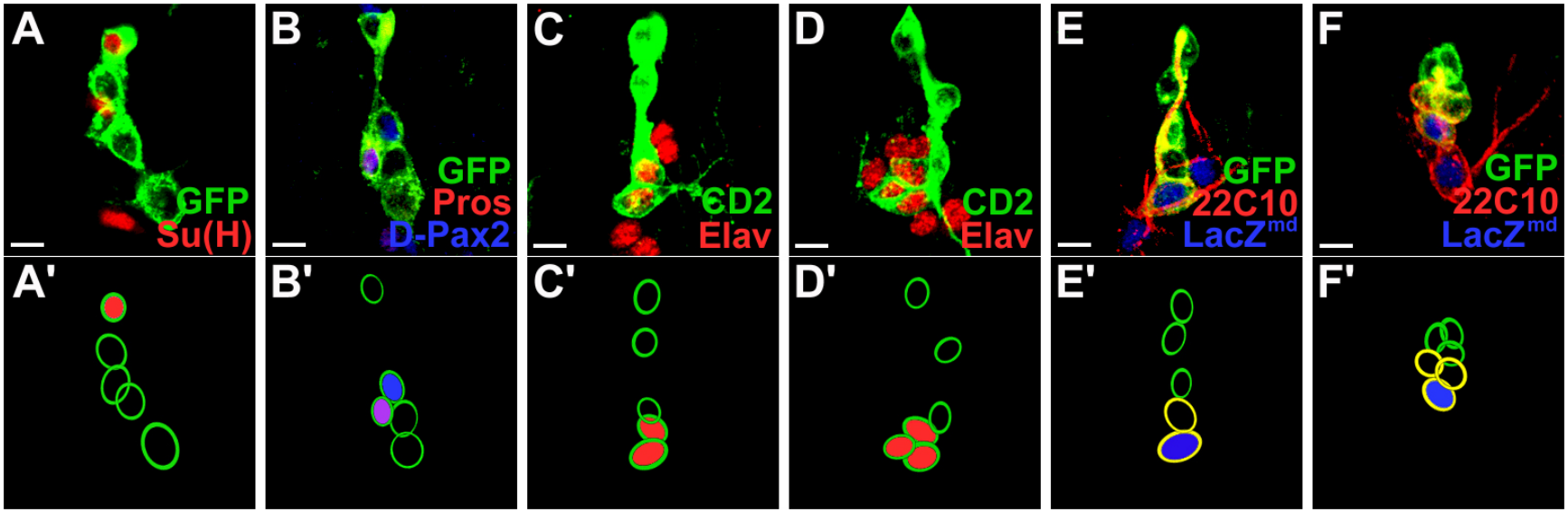

Fig. 9. Lineages of kölbchen in *Poxn*$^{\Delta M22}$ embryos gives rise to all support cells but only two or three neurons. Homozygous *Poxn*$^{\Delta M22}$ embryos were treated either with heat shock at 3-4 hours AEL (A, B, E, and F) or without heat shock (C, D) and immunostained with the indicated antibodies. Representative GFP- or CD2-expressing clones that correspond to the entire lineage of kölbchen are shown. In *Poxn*$^{\Delta M22}$ embryos, the kölbchen lineage gives rise to one socket cell that expresses Su(H) (A), one shaft cell that expresses D-Pax2 and one sheath cell that expresses both D-Pax2 and Pros (B), and two (C, E) or three (D, F) neurons. The most ventral neuron is the md neuron (E, F). **(A'-F')** Schematic representations of the clones in the corresponding panels A-F. Scale bars are 5 µm.
Genotypes: (A-D) *y w hs-flp*; *Poxn*$^{\Delta M22}$ *UAS>CD2, y*$^{+}$*>CD8:GFP*; *Poxn-Gal4*^{ups1f}*/TM6B*; (E, F) *y w hs-flp*; *Poxn*$^{\Delta M22}$ *UAS>CD2, y*$^{+}$*>CD8:GFP/Poxn*$^{\Delta M22}$ *E7-2-36*; *Poxn-Gal4*^{ups1f}*/TM6B*.

type of clones / hs at [hours AEL]	all cells	socket & shaft	sheath & neurons	socket	shaft	sheath & one bipolar neurons	md alone or md & one bipolar neuron
3 – 4	37	11	15	0	0	0	0
4 – 5	11	20	28	0	0	0	0
5 – 6	2	12	13	6	4	9	9

Table 3. Numbers and types of clones scored in the kölbchen lineage when *Poxn*$^{\Delta M22}$ embryos were heat-shocked at different developmental times (time intervals correspond to development at 25°C).

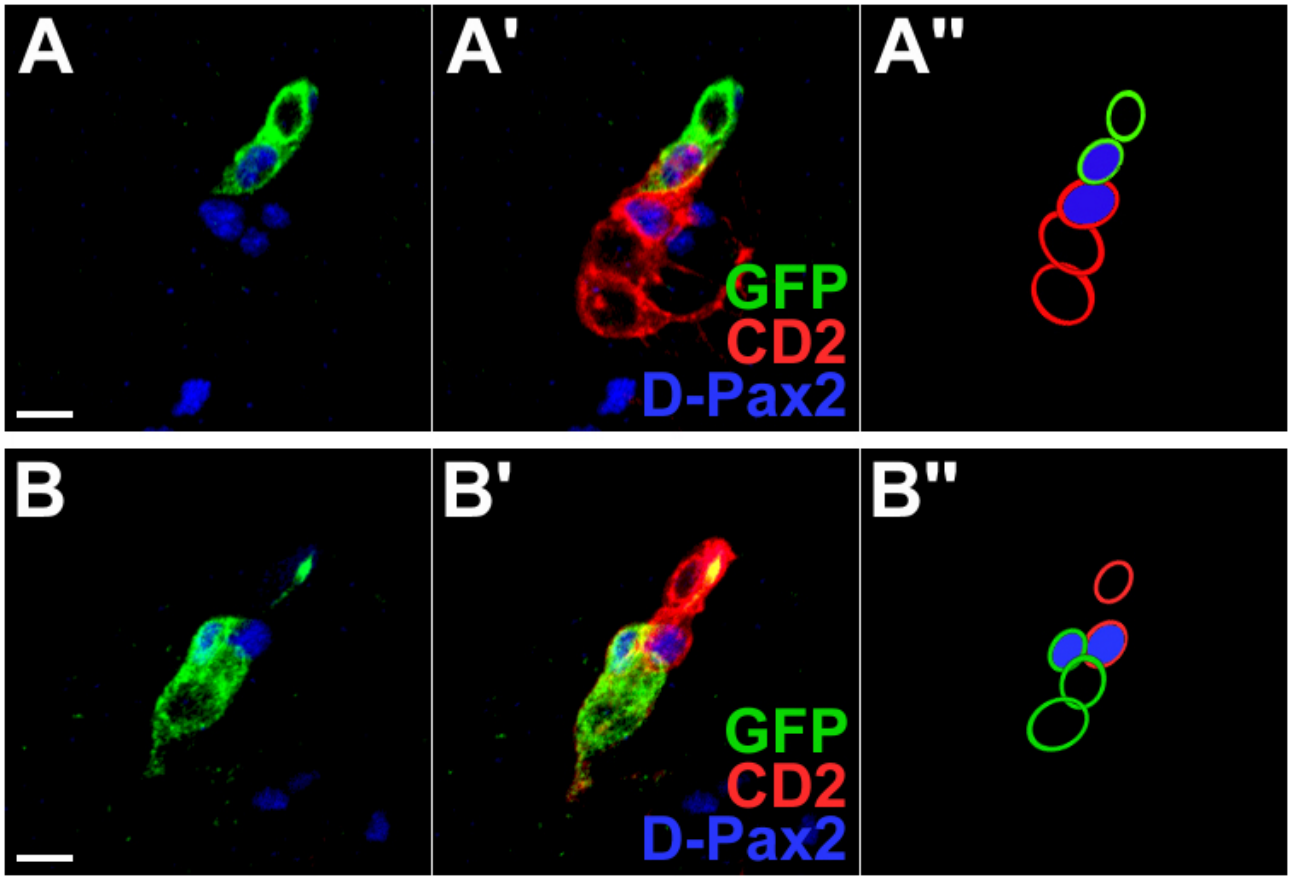

Fig. 10. Representative clones induced at 4-5 hours AEL in the kölbchen lineage of *Poxn*$^{\Delta M22}$ embryos. *Poxn*$^{\Delta M22}$ embryos were subjected to heat shock at 4-5 hours AEL and immunostained with the indicated antibodies. Lineages that consisted of five cells are shown. Two types of GFP-expressing clones were observed, which consist of either the socket and the shaft cell (A, A') or the sheath cell and two neurons (B, B'). **(A''-B'')** Schematic representations of the clones in the corresponding panels A'-B'. Scale bars are 5 µm.
Genotypes: *y w hs-flp*; *Poxn*$^{\Delta M22}$ *UAS>CD2, y*$^{+}$*>CD8:GFP*; *Poxn-Gal4*^{ups1f}*/TM6B*

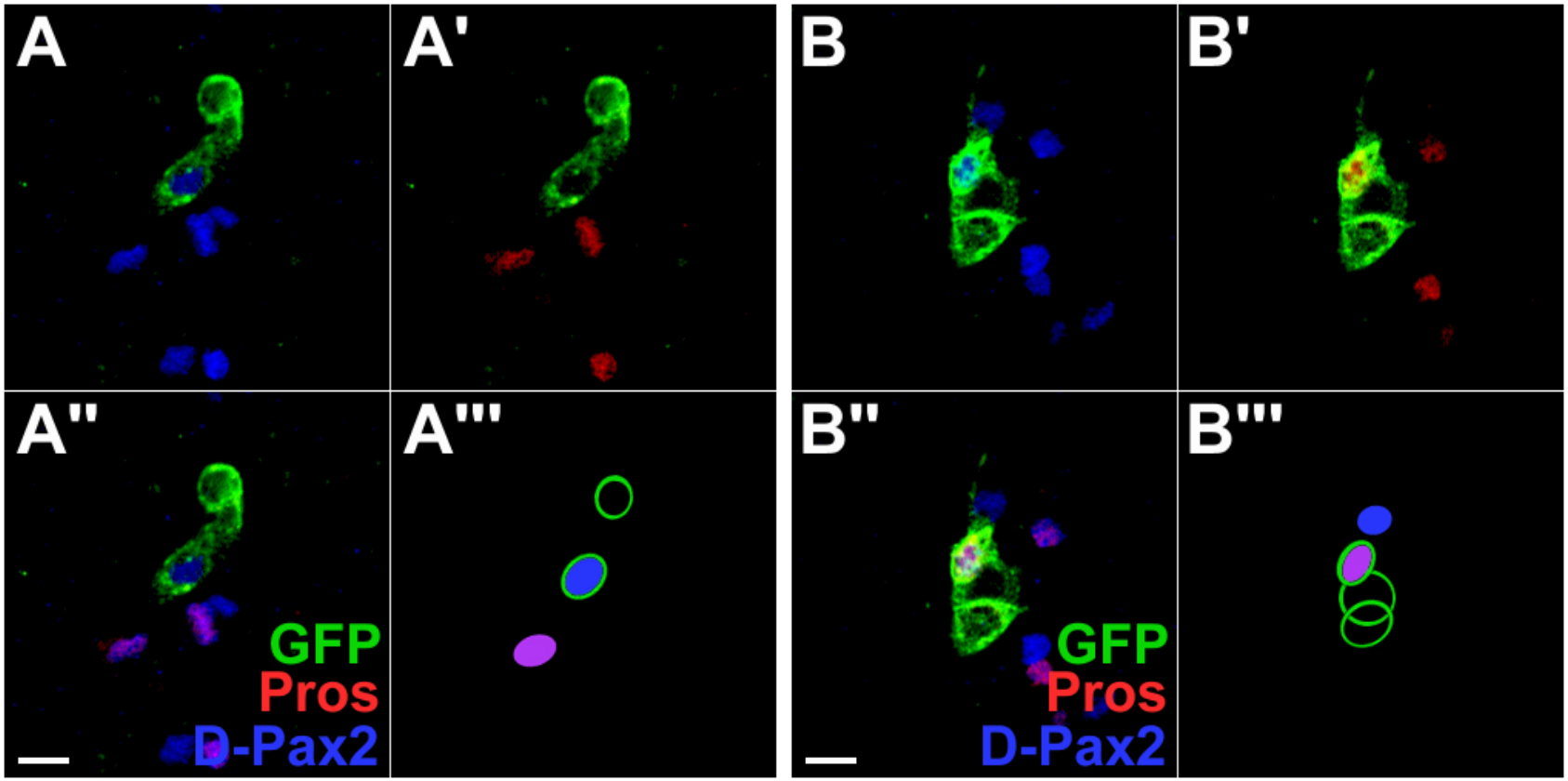

Fig. 11. Representative clones induced at 4-5 hours AEL in the kölbchen lineage of *Poxn*$^{\Delta M22}$ embryos. *Poxn*$^{\Delta M22}$ embryos were heat-shocked at 4-5 hours AEL and immunostained with the indicated antibodies. Two types of GFP-expressing clones were observed in lineages that consisted of five cells. One type consists of a socket cell, which is located most dorsally and does not express D-Pax2 and Pros, and a shaft cell that expresses only D-Pax2 but not Pros (A-A''). The other type consists of a sheath cell, which expresses both D-Pax2 and Pros, and more ventrally located neurons (B-B''). **(A'''-B''')** Schematic representations of the clones in the corresponding panels A''-B''. Scale bars are 5 µm. Genotypes: *y w hs-flp*; *Poxn*$^{\Delta M22}$ *UAS>CD2, y*$^{+}$*>CD8:GFP*; *Poxn-Gal4*^{ups1f}*/TM6B*

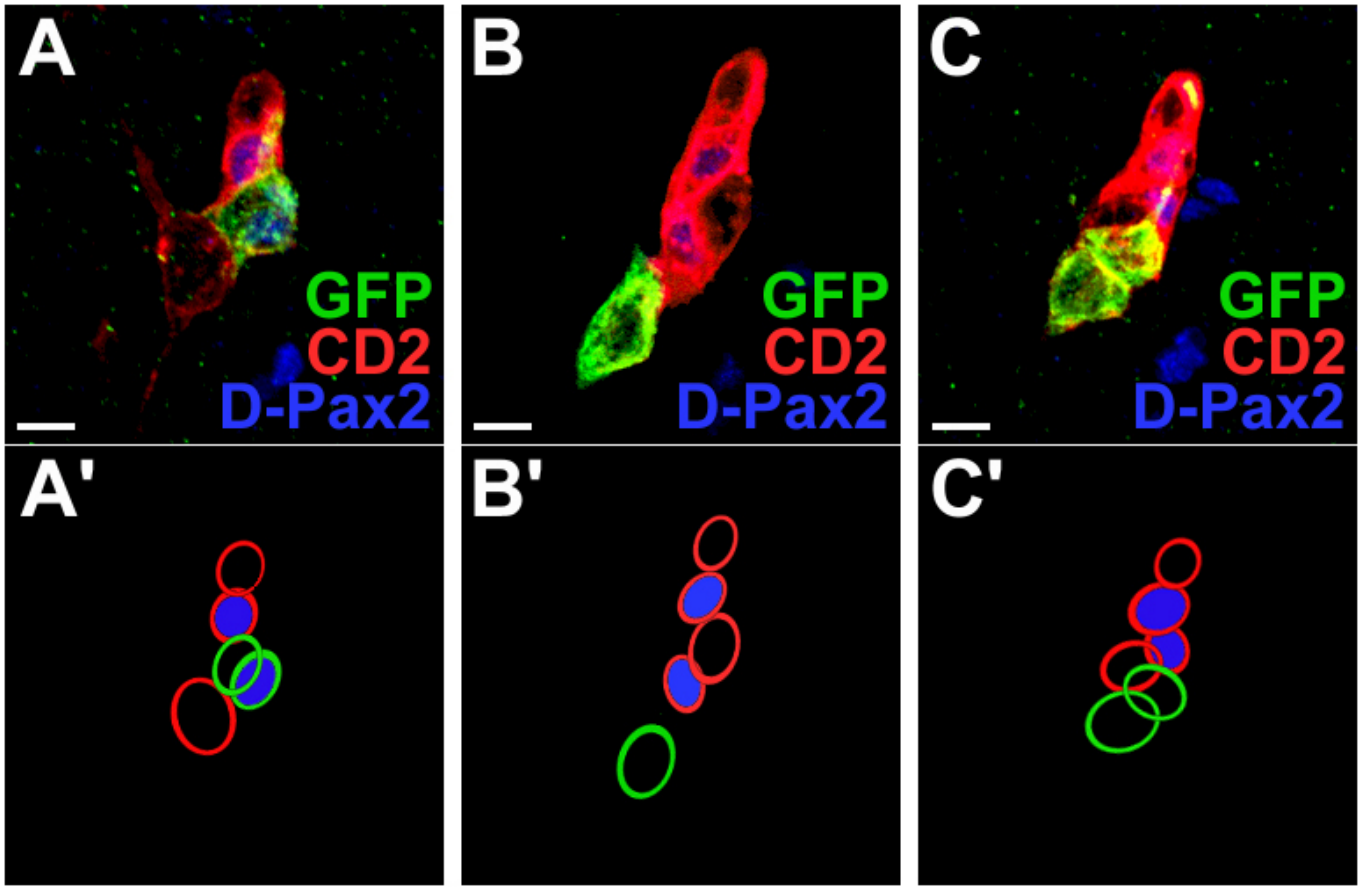

Fig. 12. Representative clones induced at 5-6 hours AEL in the kölbchen lineage of *Poxn*$^{\Delta M22}$ embryos. *Poxn*$^{\Delta M22}$ embryos were heat-shocked at 5-6 hours AEL and stained with the indicated antibodies. GFP-expressing clones induced in the lineages that consisted of five cells (A, B) or six cells (C) are shown. Clones represent the sheath cell and one bipolar neuron (A), the md neuron alone when there are five cells in the lineage (B), and one bipolar neuron and the md neuron when there are six cells in the lineage (C). **(A'-C')** Schematic representations of the clones in the corresponding panels A-C. Scale bars are 5 µm.
Genotypes: *y w hs-flp*; *Poxn*$^{\Delta M22}$ *UAS>CD2, y*$^{+}$*>CD8:GFP*; *Poxn-Gal4*^{ups1f}*/TM6B*.

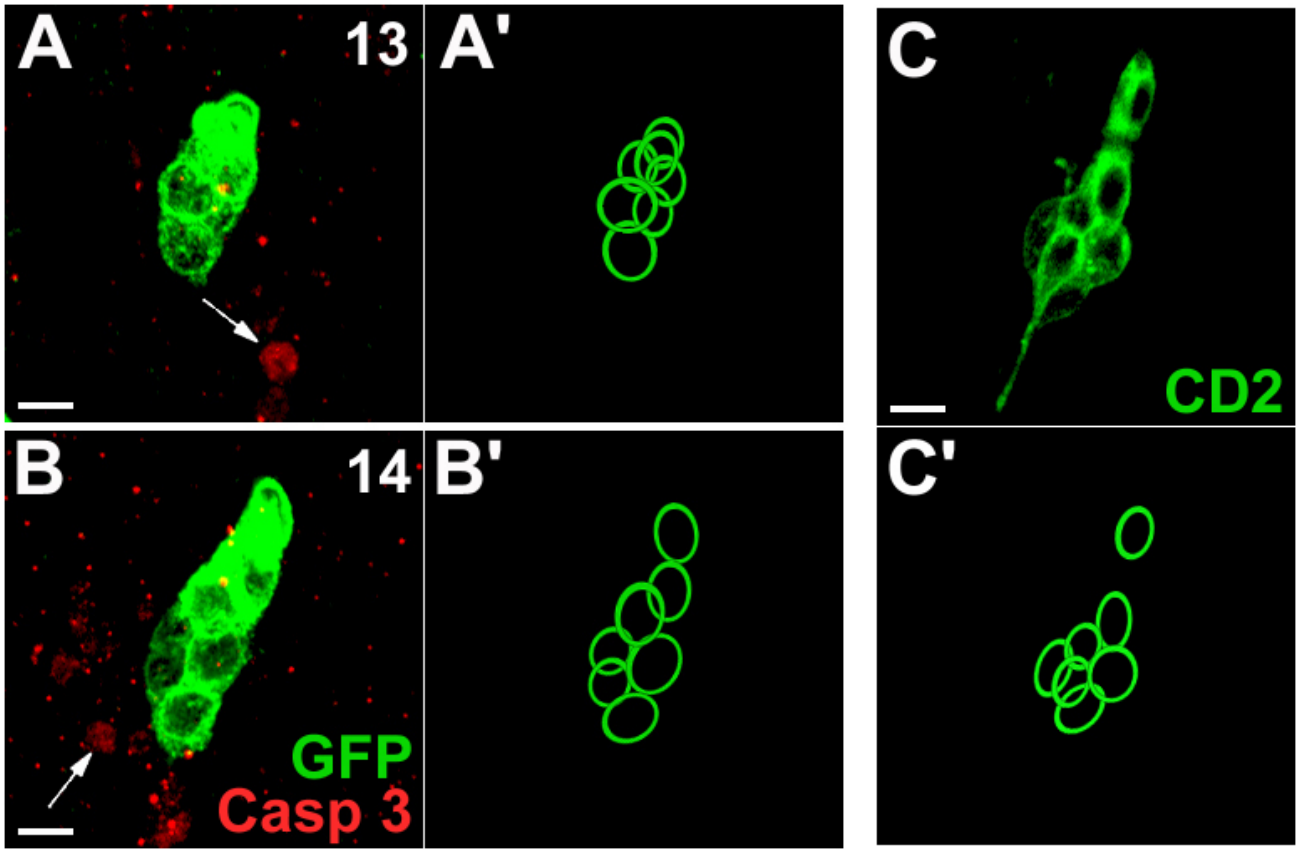

Fig. 13. No evidence for apoptosis in the kölbchen lineage in wild-type embryos. **(A, B)** Embryos between stage 12 and 15 were collected and stained with the indicated antibodies. GFP-expressing cells representing the entire kölbchen lineage in embryos of stage 13 (A) and stage 14 (B) are shown. Note that none of the GFP-expressing cells expresses activated Caspase 3 (Casp 3), a marker for apoptosis. Arrows indicate apoptotic cells in adjacent tissues. **(C)** CD2-positive cells represent the kölbchen lineage. The lineage consists of seven cells, as in wild-type, and no extra cells are observed in the kölbchen lineage when apoptosis is inhibited by the expression of the anti-apoptotic protein P35. **(A'-C')** Schematic representations of the clones in the corresponding panels A-C. Scale bars are 5 µm.
Genotypes: (A, B) *y w*; *UAS-CD8:GFP*; *Poxn-Gal4*^{ups1f}*/TM3*; (C) *y w hs-flp/*(*w* or *Y*); *UAS>CD2, y*$^{+}$*>CD8:GFP/UAS-p35*; *Poxn-Gal4*^{ups1f}*/+*.

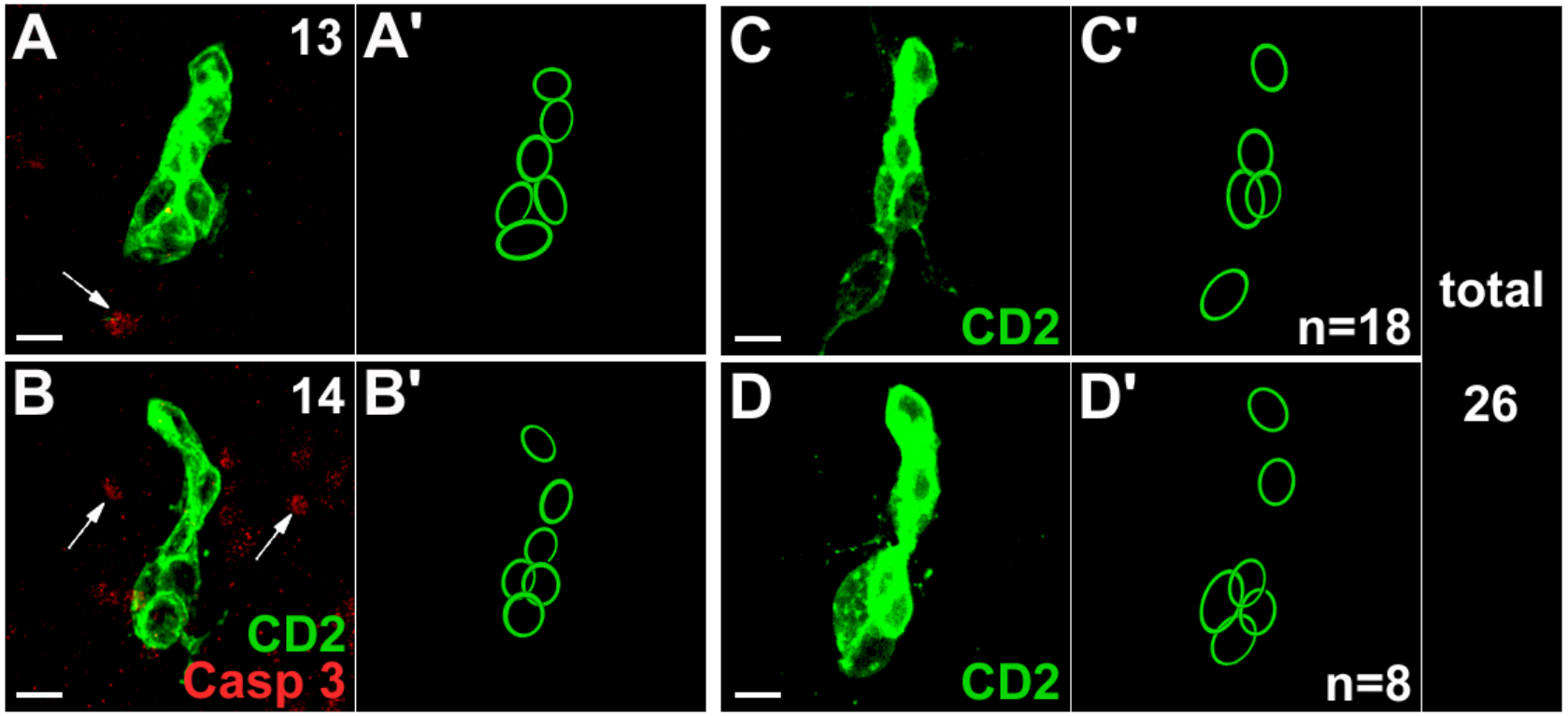

Fig. 14. No evidence for apoptosis in the kölbchen lineage in *Poxn*$^{\Delta M22}$ embryos. **(A, B)** *Poxn*$^{\Delta M22}$ embryos between stages 12 and 15 were collected and stained with the indicated antibodies. CD2-expressing cells representing the kölbchen lineage that consisted of six cells in embryos of stage 13 (A) and stage 14 (B) are shown. Note that none of the CD2-expressing cells expresses the apoptosis marker activated Caspase 3 (Casp 3). Arrows indicate apoptotic cells in adjacent tissues. **(C, D)** CD2-expressing cells represent the two alternative kölbchen lineages in *Poxn*$^{\Delta M22}$ embryos when apoptosis is inhibited during the development of kölbchen. No extra cells were observed when apoptosis is inhibited by the expression of P35. The ratio of the two alternative lineages remains the same as observed in embryos without P35 expression. **(A'-D')** Schematic representations of the clones in the corresponding panels A-D. Scale bars are 5 μm.
Genotypes: (A, B) *y w hs-flp*; *Poxn*$^{\Delta M22}$ *UAS>CD2, y*$^{+}$*>CD8:GFP*; *Poxn-Gal4*^{ups1f}*/TM6B*; (C, D) *y w hs-flp*; *Poxn*$^{\Delta M22}$ *UAS>CD2, y*$^{+}$*>CD8:GFP/Poxn*$^{\Delta M22}$ *UAS-p35*; *Poxn-Gal4*^{ups1f}*/TM6B*.

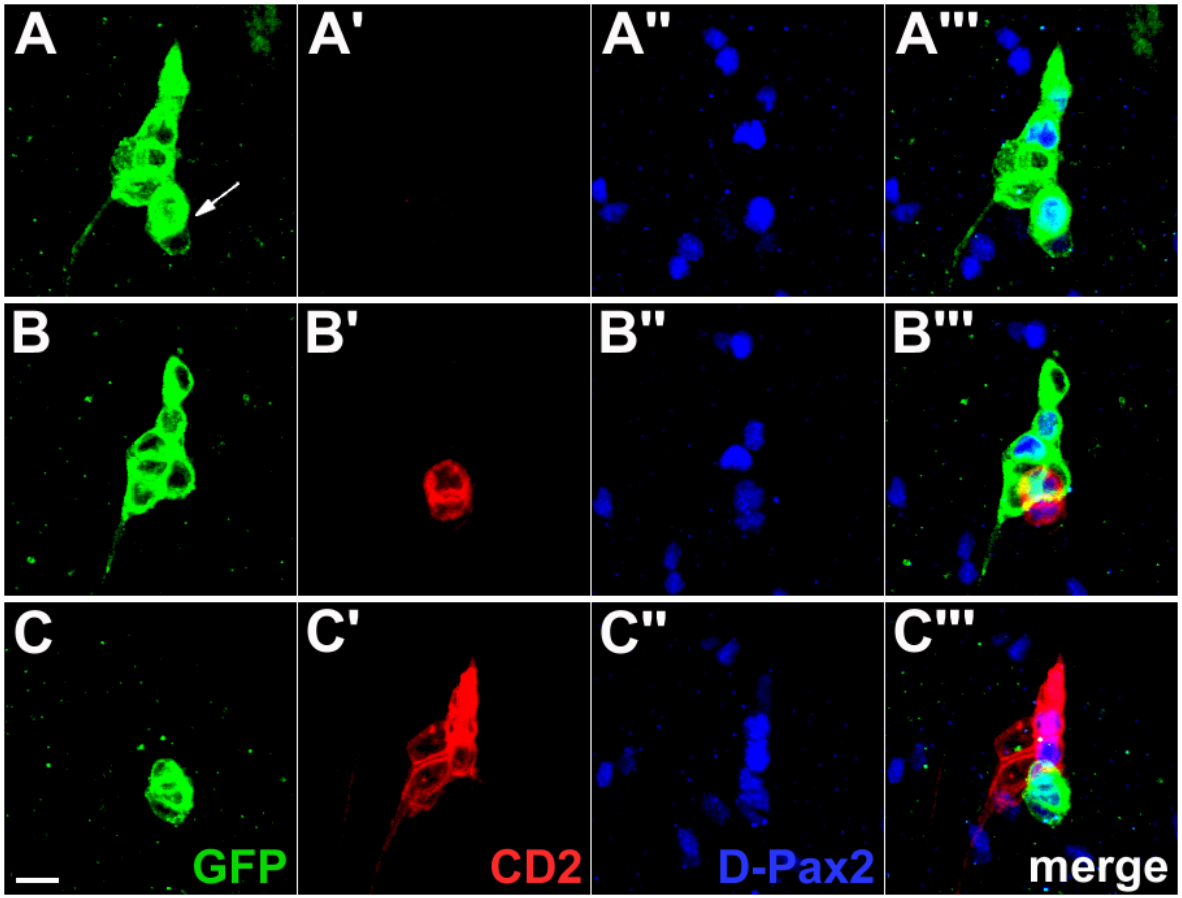

Fig. 15. Papilla 6 and a presumptive epidermal gland are derived from a single precursor. Wild-type embryos were subjected to heat shock at 3-4 hours AEL and immunostained with the indicated antibodies. When all cells generated by the lineage were labeled by GFP (A-A'''), in addition to the p6 cells, most clones contain another three cells (arrow in A) proposed to form an epidermal gland (Orgogozo and Schweisguth, 2004). Meanwhile, we also observed GFP-positive clones corresponding to either the p6 cells (B-B''') or the presumptive epidermal gland cells (C-C'''). Scale bars are 5 µm.
Genotypes: *y w hs-flp*; *UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *Poxn-Gal4*^{ups1f}*/TM6B*.

type of clones / hs at [hours AEL]	p6 cells and eg cells	eg cells	p6 cells	support cells	neurons	socket	shaft & sheath	two bipolar neurons	md
2 – 3	94	10	16	0	0	0	0	0	0
3 – 4	56	23	28	0	0	0	0	0	0
4 – 5	4	33	32	2	1	0	0	0	0
5 – 6	0	n.d.	8	33	35	1	8	2	0
6 – 7	0	n.d.	0	8	6	16	49	36	15

Table 4. Numbers and types of clones scored in the p6 lineage when wild-type embryos were heat-shocked at different developmental times. eg cells are three cells proposed to form an epidermal gland (Orgogozo and Schweisguth, 2004). Number of clones, induced in the eg lineage by heat shock at 5-6 or 6-7 hours AEL, were not determined (n.d.).

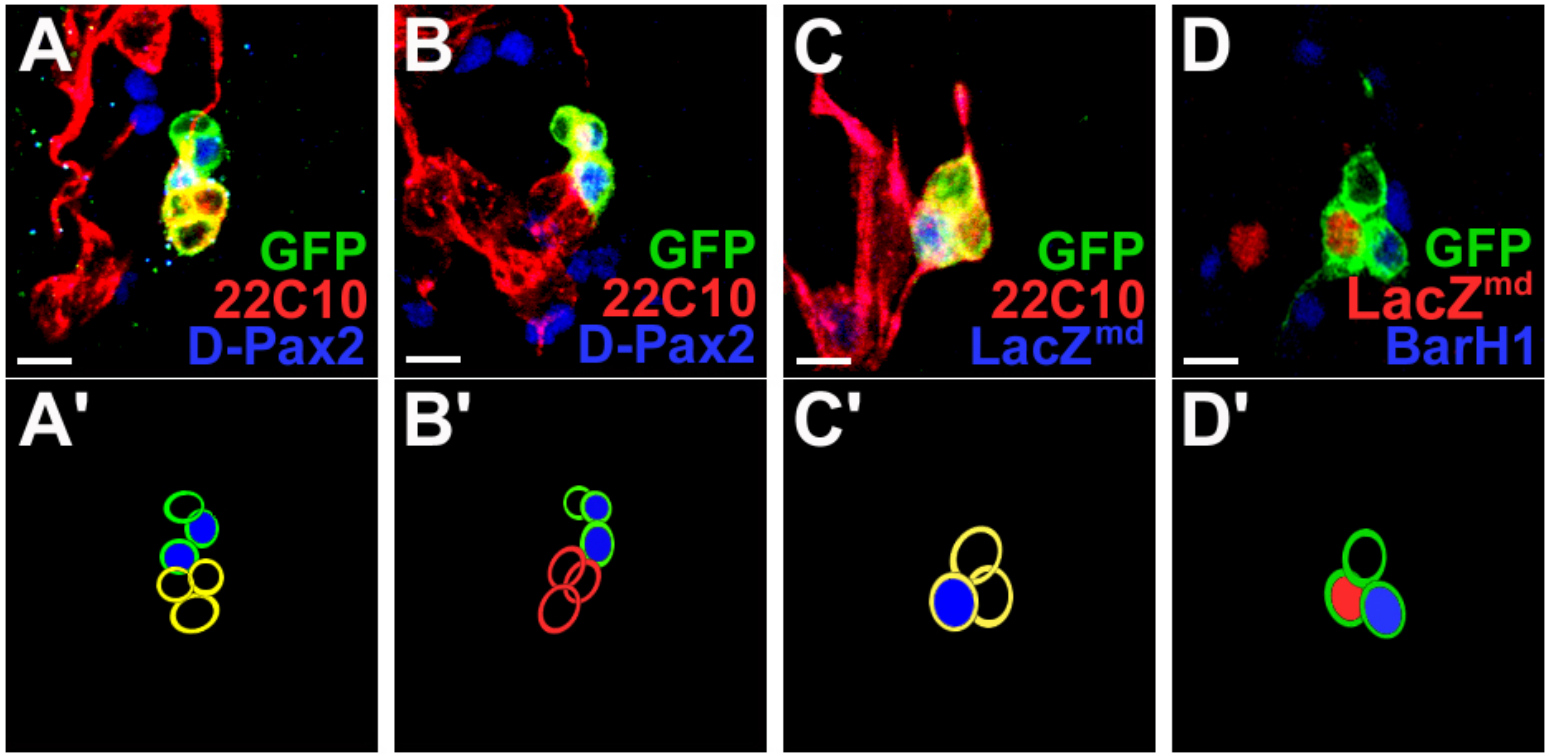

Fig. 16. Representative clones induced at 5-6 hours AEL in the p6 lineage. Wild-type embryos subjected to heat shock at 5-6 hours AEL and stained with the indicated antibodies. GFP-expressing clones representing the entire p6 lineage (A) or a subset of the p6 lineage (B-D) are shown. **(A)** The p6 lineage gives rise to six cells expressing GFP (green), including one socket cell that is located most dorsally, one shaft and one sheath cell that express D-Pax2 (blue), and three neurons that are 22C10-positive (red). Cells expressing GFP and 22C10 are labeled yellow. **(B-D)** When embryos were heat-shocked at 5-6 hours AEL, mainly two types of GFP-expressing clones in the p6 lineage were observed, which consisted of either all support cells (B) or all neurons (C, D). One of the three neurons is an md neuron, as it expresses the md neuron marker LacZ (C, D). Of the two bipolar neurons, only one expresses BarH1 (D). **(A'-D')** Schematic representations of the clones in the corresponding panels A-D. Scale bars are 5 µm.

Genotypes: (A, B) *y w hs-flp*; *UAS>CD2, y^{+}>CD8:GFP/CyO*; *Poxn-Gal4^{ups1f}/TM6B*; (C, D) *y w hs-flp*; *E7-2-36 UAS>CD2, y^{+}>CD8:GFP/CyO*; *Poxn-Gal4^{ups1f}/TM6B*.

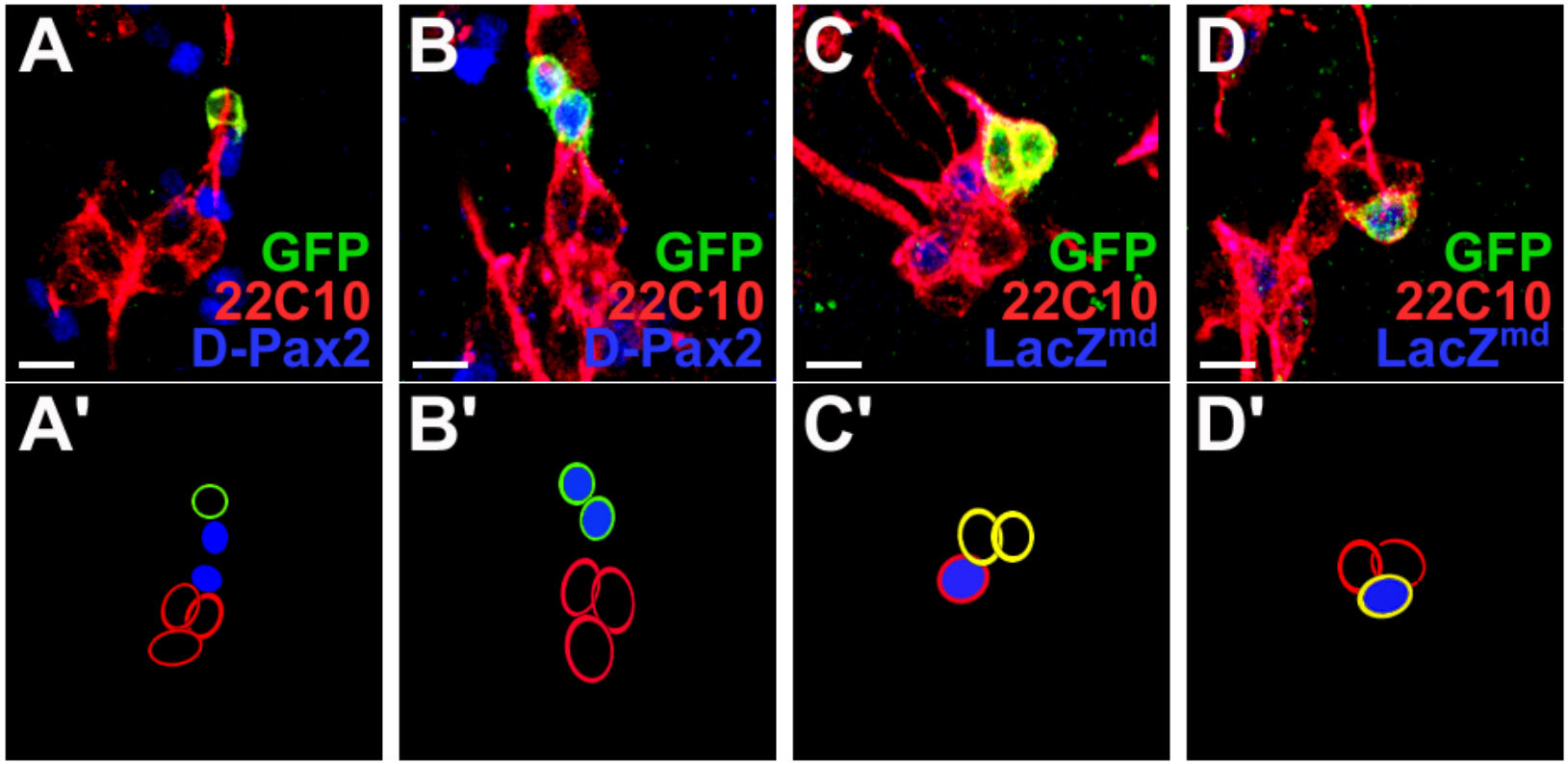

Fig. 17. Representative clones induced at 6-7 hours AEL in the p6 lineage. Wild-type embryos were subjected to heat shock at 6-7 hours AEL and stained with the indicated antibodies. GFP-expressing clones representing the socket cell (A), the shaft and the sheath cell (B), the two bipolar neurons (C), and the md neuron (D) are shown. **(A'-D')** Schematic representations of the clones in the corresponding panels A-D. Scale bars are 5 μm.

Genotypes: (A, B) *y w hs-flp*; *UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *Poxn-Gal4*^{ups1f}*/TM6B*; (C, D) *y w hs-flp*; *E7-2-36 UAS>CD2, y*$^{+}$*>CD8:GFP/CyO*; *Poxn-Gal4*^{ups1f}*/TM6B*.

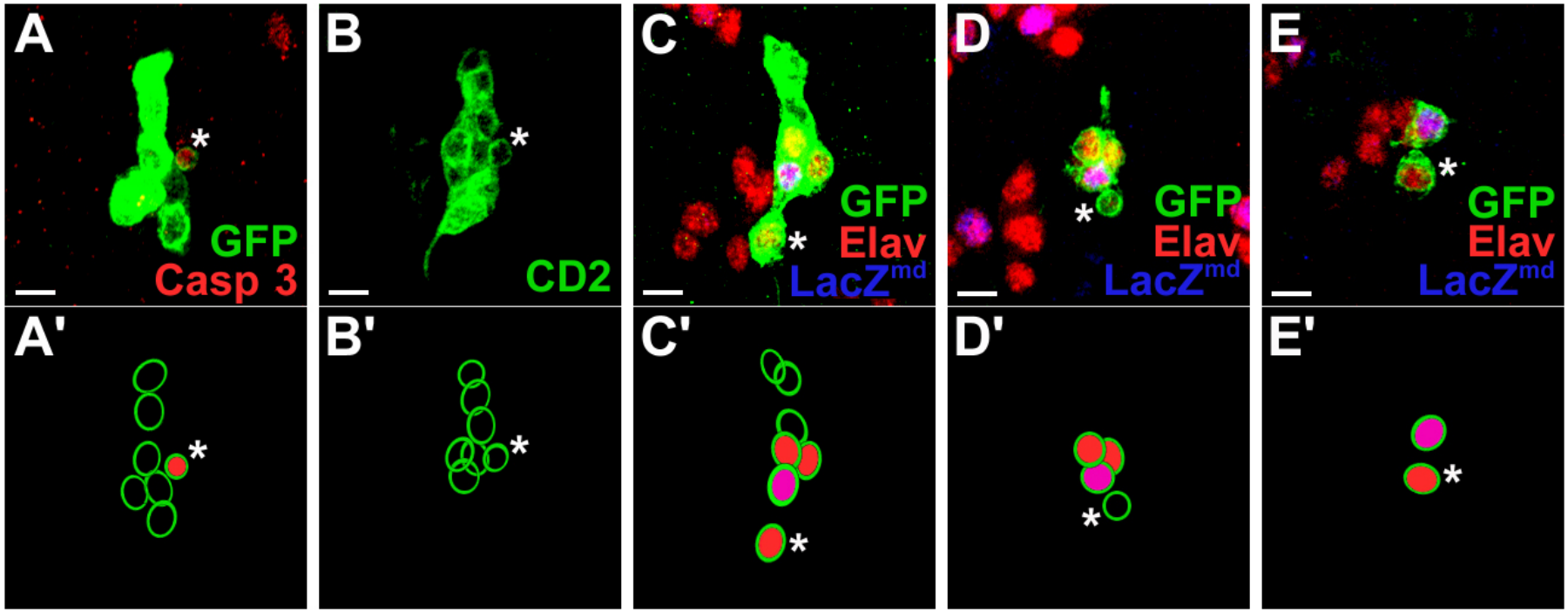

Fig. 18. One cell from the p6 lineage undergoes apoptosis. **(A)** GFP-expressing cells representing the p6 lineage and the epidermal gland lineage in a stage 13 embryo are shown. Note that an extra GFP-expressing cell is labeled by the apoptotic cell marker activated Caspase 3 (Casp 3, asterisk). **(B-E)** Apoptosis is inhibited by the expression of P35, which generates an extra cell (asterisks). Which of the ten cells is the extra cell in (B) is uncertain. This extra cell is derived from the p6 lineage, as shown in a clone representing only the p6 lineage (C). In many cases, this apoptotic cell expresses the neuronal marker Elav (C). When clones were induced at 5-7 hours AEL, we found that the apoptotic cell is generated from the pIIb cell with other neurons (D) and is derived from the same precursor cell as the md neuron (E). **(A'-E')** Schematic representations of the clones in the corresponding panels A-E. Note that the epidermal gland cells in A and B are not illustrated in A' and B'. Scale bars are 5 µm.

Genotypes: (A) *y w*; *UAS-CD8:GFP*; *Poxn-Gal4*^{ups1f}*/TM6B*; (B) *y w hs-flp/(w* or *Y)*; *UAS>CD2, y*$^{+}$*>CD8:GFP/UAS-p35*; *Poxn-Gal4*^{ups1f}/+; (C-E) *y w hs-flp/(w* or *Y)*; *E7-2-36 UAS>CD2, y*$^{+}$*>CD8:GFP/UAS-p35*; *Poxn-Gal4*^{ups1f}/+.

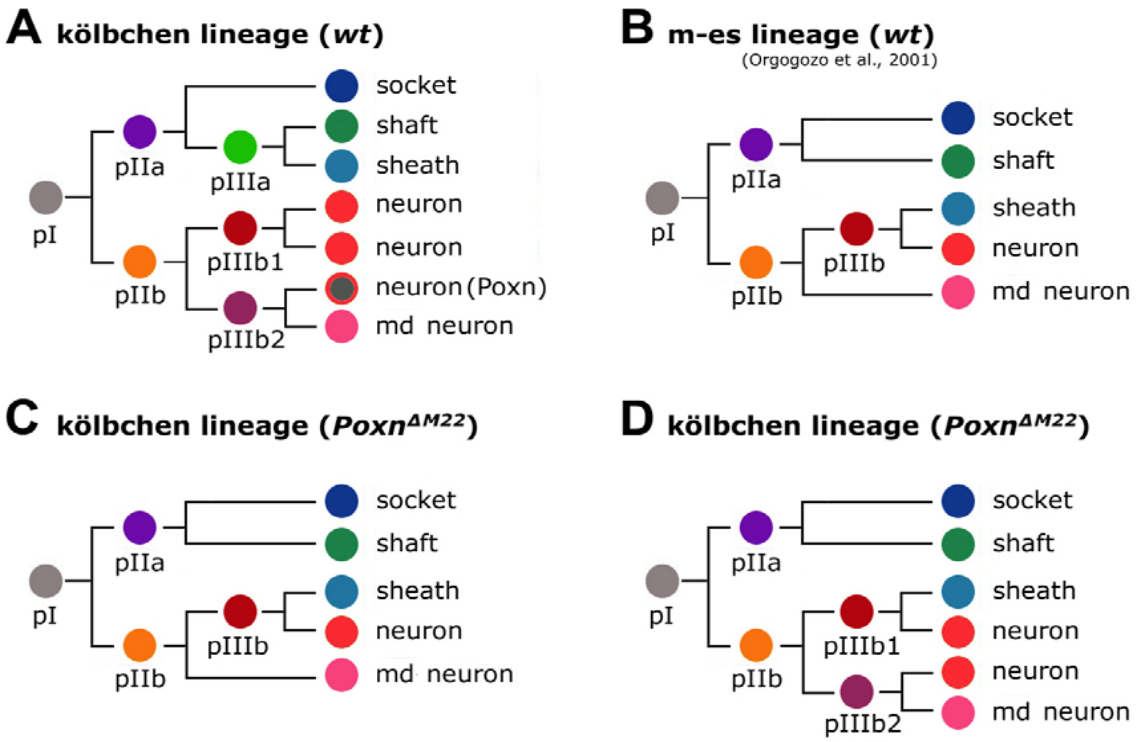

Fig. 19. Models for the kölbchen lineage in wild-type and *Poxn*$^{\Delta M22}$ mutant embryos. **(A)** Cell lineage of kölbchen in wild-type embryos. Different cell types are marked by different colors. The SOP cell, or pI, divides to give rise to two secondary cells, pIIa and pIIb; the pIIa cell divides to generate the socket cell and pIIIa cell, which is the precursor of the shaft cell and the sheath cell; the pIIb cell divides to give rise to the pIIIb1 and pIIIb2 cells; the pIIIb1 cell is the precursor of two bipolar neurons, and the pIIIb2 cell is the precursor of the md neuron and the third bipolar neuron that expresses Poxn during late embryogenesis. **(B)** Cell lineage of m-es organs in wild-type embryos (Orgogozo et al., 2001). In this lineage, the sheath cell is derived from the pIIb cell. **(C, D)** In *Poxn*$^{\Delta M22}$ mutants, there are two alternative kölbchen lineages that consist of either five cells (C) or six cells (D). The kölbchen lineage gives rise to a reduced number of cells (neurons), and the cell division pattern is changed: the sheath cell is derived from the pIIb cell. When the lineage gives rise to five cells (C), the cell division pattern is the same as that in the m-es lineage (B); when the lineage gives rise to six cells, the cell division pattern is similar to the m-es lineage, except that the pIIIb2 cell undergoes an additional cell division (D).

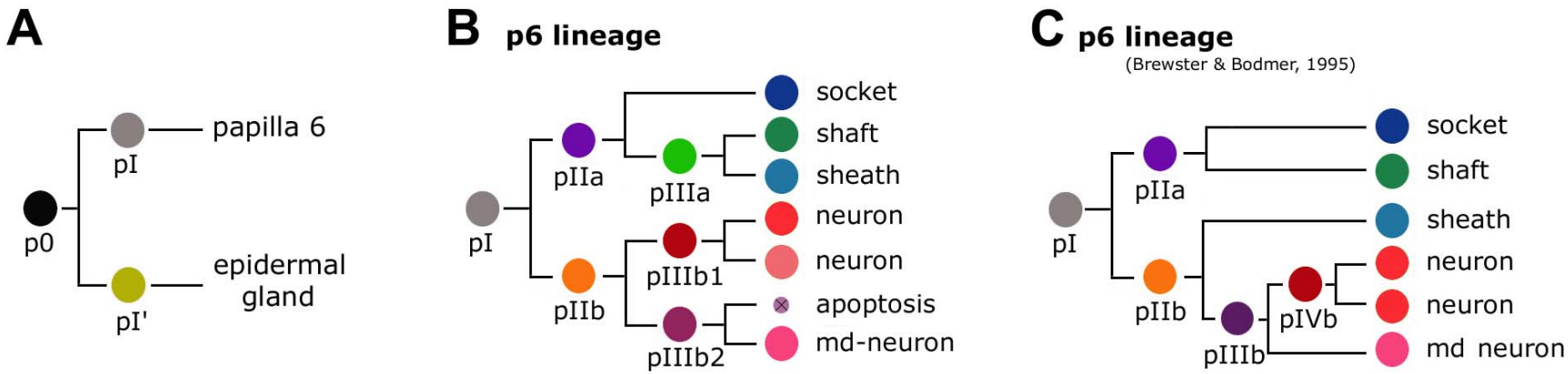

Fig. 20. Models for the p6 lineage in wild-type embryos. **(A)** The SOP cell of p6, pI, and the precursor cell of an epidermal gland, pI', are derived from the same precursor p0. **(B)** Cell lineage of p6 in wild-type embryos from this study. Different cell types are marked by different colors. The SOP cell, pI, divides to give rise to two secondary cells, pIIa and pIIb; the pIIa cell divides to generate the socket cell and pIIIa cell, which is the precursor of the shaft cell and sheath cell; the pIIb cell divides to give rise to pIIIb1 and pIIIb2 cells; the pIIIb1 cell is the precursor of two bipolar neurons, only one of which expresses BarH1, while the pIIIb2 cell divides to generate an md neuron and a cell that undergoes apoptosis soon after birth. **(C)** Model for the p6 lineage proposed previously (Brewster and Bodmer, 1995).

Chapter 3

Conclusions

In previous studies, it has been shown that *Poxn* plays an important role in the determination of larval p-es organs as well as their homologs in adults, the chemosensory bristles. However, how *Poxn* exerts its function during these processes remains elusive. In this study, we described the detailed analysis of cell lineages for two larval p-es organs, kölbchen and papilla 6. Surprisingly, these two p-es lineages are distinct from any cell lineage reported previously. Most strikingly, the sheath cell is generated from the pIIa cell, one of the two secondary precursors, together with the socket and the shaft cell, while all neurons are produced from the other secondary precursor, the pIIb cell. In homozygous $Poxn^{\Delta M22}$ embryos, the kölbchen lineage gives rise to a reduced number of cells and the cell division pattern is altered. Taken together, our results indicate that *Poxn* plays an important role in the specification of the cell lineage during the development of p-es organs in *Drosophila* larvae.

The kölbchen lineage is a novel cell lineage

We have described a novel cell lineage for kölbchen. The kölbchen lineage gives rise to seven cells: three support cells, consisting of a socket, a shaft, and a sheath cell, three bipolar neurons, and an md neuron. The SOP cell divides to generate two secondary precursors, the pIIa and pIIb cells. The pIIa cell divides to give rise to all support cells, and the pIIb cell is the precursor of all neurons. The most unexpected finding in this lineage is that the sheath cell is generated from the secondary precursor pIIa, whereas in all previously reported lineages, the sheath cell is produced from the pIIb cell. As the cell fates are determined by the asymmetric segregation of cell fate determinant proteins and cell-cell communications between the sibling cells, it would be interesting

to investigate in future experiments how the cell fate determinants are localized during cell divisions and how Notch signaling is regulated in the kölbchen lineage.

The p6 lineage is similar to the kölbchen lineage but significantly different from a previously proposed lineage

The cell lineage of p6 has been analyzed in a previous study, which claimed that its pattern of cell divisions is similar to that of the m-es lineage. However, when we re-analyzed the p6 lineage using our method, we found that the p6 lineage is similar to the kölbchen lineage but shows significant differences to the previously proposed model. The p6 lineage gives rise to six cells: three support cells, two bipolar neurons, and an md neuron. All support cells are generated from the same secondary precursor, the pIIa cell, and all neurons are generated from the pIIb cell. We also found that the SOP cell of p6 and the precursor of a putative epidermal gland are derived from the same precursor cell that expresses *Poxn*. Furthermore, we observed that one cell of the p6 lineage undergoes apoptosis shortly after birth.

It is interesting to note that apoptosis appears to be specific for the p6 lineage, as we did not observed any cell death in the kölbchen lineage. It has been shown recently that the Hox genes *Antp* and *Ubx* regulate segment-specific apoptosis of differentiated motoneurons in the ventral nerve cord of *Drosophila* embryos. *Ubx* is required to activate segment-specific apoptosis in these cells, while their survival depends on *Antp*. Whether the apoptosis events observed in the p6 lineage is regulated through the same mechanism by Hox genes remains to be investigated.

***Poxn* is necessary for the specification of the p-es lineages**

In homozygous $Poxn^{\Delta M22}$ embryos, the kölbchen lineage gives rise to a reduced number of cells, and the cell division pattern is altered. We observed two alternative lineages in the absence of

Poxn. About 65% of them produce five cells, and the cell division pattern is the same as that of the m-es lineage, which suggest a complete transformation of p-es organs into m-es organs. Surprisingly, we also found that 35% of the lineage gives rise to six cells, including two bipolar neurons. However, the cell division pattern of this type of lineage is also transformed to an m-es-like pattern. These alterations of the cell division pattern in the kölbchen lineage suggest that *Poxn* plays a crucial role in the specification of the p-es lineages.

As *Poxn* also plays an essential role during the development of the poly-innervated chemosensory sensilla in adult flies, it would be interesting, from an evolutionary point of view, to test whether these homologs of the larval p-es organs develop through similar lineages. It is still not clear whether *Poxn* is sufficient for the determination of the p-es lineage. To perform such an analysis, *Poxn* has to be ectopically expressed in the SOP cells and all their progenies of the m-es organs, in a way that is the same as the wild-type expression pattern of *Poxn* in the p-es organs. For instance, by tests using a transgene that expresses *Poxn* directly under the control of the promoter and appropriate enhancer of *D-Pax2* to avoid the temporal delay caused by the Gal4/UAS system.

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Curriculum Vitae

YANRUI JIANG

email: yanrui.jiang@molbio.uzh.ch

Date of Birth:	16 March 1977
Place of Birth:	Jilin, China
Nationality:	China
Marital Status:	Married

Education

07.2003 – present	Graduate student, Institute of Molecular Biology and Ph.D. Program in Molecular Life Sciences University of Zürich Winterthurerstrasse 190 CH-8057 Zürich, Switzerland
09.1995 – 07.1999	B. Sc., Genetics Institute of Genetics, School of Life Sciences Fudan University No.220 Handan Road 200433 Shanghai, China
09.1992 – 07.1995	Jilin No.1 High School 132012 Jilin City, Jilin Province, China

Professional Experience

11.2002 – 07.2003	R&D Department Supervisor National Human Genome Center at Beijing No. 3-707 North Yongchang Road, BDA 100176 Beijing, China
11.2001 – 10.2002	Guest Scientist Roche Center for Medical Genomics (RCMG) F. Hoffmann-La Roche ltd. Grenzacherstrasse 124 CH-4070 Basel, Switzerland
05.2000 – 10.2001	Head of Sequencing Lab and QC Supervisor National Human Genome Center at Beijing No. 3-707 North Yongchang Road, BDA 100176 Beijing, China

08.1999 – 04.2000 Research Assistant
Institute of Genetics, School of Life Sciences
Fudan University
No.220 Handan Road
200433 Shanghai, China

Conference Presentations

Jiang, Y.R., Boll, W., and Noll, M. Novel cell lineage in a larval sensory organ controlled by *Pox neuro*. The 12th European *Drosophila* Neurobiology Conference. September, 2008. Würzburg, Germany.

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*Lists of contributors and their affiliations appear in the online Supplementary Information.

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